FACTORS AFFECTING THE GROWTH AND SURVIVAL OF *SALMONELLA* AND SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ON MICROGREENS

A Dissertation

by

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ABSTRACT

Microgreens are an emerging commodity within the vegetable group with high consumer demand due to their high nutritional value and flavor attributes. To date, there have been no reported outbreaks linked to the contamination of microgreens. In addition, there are only limited experimental data available on the microbiological safety of microgreens. Therefore, not enough knowledge exists on the safety of microgreens. Food safety concerns have been expressed surrounding the similarities that are thought to exist between sprouts and microgreens. Sprouts represent a high food safety concern because the conditions under which they are produced (temperature, time, humidity, and nutrient availability) are ideal for the proliferation of foodborne pathogens. Sprouts have been linked to numerous foodborne outbreaks. Since contaminated seeds have been identified as the main source of pathogenic bacteria in sprout-related outbreaks, this could be also true for microgreens.

The first objective of this research focused on analyzing the growth and behavior of *Salmonella* and STEC on alfalfa sprouts and microgreens obtained from inoculated seed with an initial concentration of 5 log CFU/g of each organism. Results indicated sprouts contained higher concentrations (8.0 log CFU/g) of target organisms compared to microgreens (7.0 log CFU/g) ($P<0.05$). The second objective of this research was to determine the effects of production practices and plant type on the growth and survival of *Salmonella* and STEC at harvesting. Results indicated that harvest period played a significant role in pathogen reduction. Broccoli, mustard, and clover microgreens had

significant reduction of *Salmonella* (0.3 - 2.3-log) and STEC (0.2-2.1-log) populations at 4 weeks compared to 2 weeks ($P<0.05$). For production practices, our results indicated that microgreen plants cut at 6.5 cm above soil surface contained significantly lower bacterial counts compared to 2.5 cm for both broccoli and clover microgreens ($P<0.05$).The final objective of this research included determining the spatial distribution of *Salmonella* Poona after inoculation on microgreens. For clover and mustard microgreens, *S.* Poona was more prevalent on inedible portions (seed coats) than the edible portions (middle shoot and leaves), indicating that seed coats were the primary source for contamination of microgreens. Overall, the findings from this research provided new insight for the production of microgreens, such as harvest period and production practices that could aid in developing food safety practices for the microgreen industry. Not only does this research provide new awareness for microgreens, but also for other vegetables that are grown from seeds.

DEDICATION

This dissertation is dedicated to my mother and father, Paula and Roger Bryant, whose support and encouragement have given me confidence to be able to pursue my goals.

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NOMENCLATURE

CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
cm	Centimeters
d	Day(s)
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FDA	United States Food and Drug Administration
FSIS	Food Safety and Inspection Service
g	Gram(s)
HUS	Hemolytic Uremic Syndrome
H	High
LIA	Lysine Iron Agar
L	Low
LSPR	Lactose Sulfite Phenol Red Rifampicin
LPS	Lipopolysaccharide
MDP	Microbiological Data Program
ml	Milliliter(s)

OM	Outer Membrane
ppm	Parts Per Million
PPP	Pentose Phosphate Pathway
Rif	Rifampicin Resistant
STEC	Shiga toxin-producing *Escherichia coli*
TSI	Triple Sugar Iron
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VH	Very High
VL	Very Low
XLD	Xylose Lysine Deoxycholate

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CHAPTER I

INTRODUCTION

Foodborne disease outbreaks related to fresh produce have a substantial health and economic impact on the U.S. population despite better hygiene and sanitation practices, scientific treatment of foods, and consumer awareness *(105)*. The incidence of outbreaks of foodborne illness continues to increase, possibly due to factors such as new production methods for fruits and vegetables, as well as manifestation of pathogens *(3, 39, 196)*. According to the Centers for Disease Control and Prevention (CDC) known and unknown pathogens have been estimated to cause approximately 48 million foodborne related illnesses, 128,000 hospitalizations, and 3,000 deaths *(188)*. According to the CDC, 864 foodborne illness outbreaks resulted in 13,246 illnesses, 712 hospitalizations, and 21 deaths in 2014 *(49)*. Of those illnesses, more than half were associated with fresh produce *(49)*. Due to increased importation of fruits and vegetables, the consumption of fresh produce per capita has increased in the U.S., as well as in other countries *(26)*. Imported fruits and vegetables from a single or commercial processor being dispersed throughout the country, may have a significant impact on the epidemiology of foodborne disease outbreaks *(26)*.

Fruits and vegetables play an important role in a well-balanced diet based on their nutritional component. Health officials encourage the consumption of fruits and vegetables due to their protection against an array of illnesses such as cardiovascular disease and cancer *(25)*. Traits such as lack of cholesterol, less saturated fat, and

antioxidants have scientifically proved to help fight these diseases. Fresh fruit and vegetable consumption has increased considerably worldwide with the help of new technologies *(124)*. These new technologies include alternative cropping systems, which address concerns with conventional agriculture practices and the use of chemical products *(139)*. Fruits and vegetables can also be home-grown or bought at local farmers' markets or retail outlets for consumption at home or restaurants *(26)*. As demands have been placed on the food market for the production of more fresh fruits and vegetables year-round, and by having a wide variety of products available, consumers now have options for foods with greater nutritional and taste benefits *(124)*. However, more emphasis is being placed on food safety guidelines during production practices of fruits and vegetables.

Fresh produce items are often produced in open fields and in close proximity to the ground. Furthermore, other production practices and environmental factors can contribute to an increase risk of contamination with human pathogens. Because of the bulk of information, indicating the limitations of produce decontamination procedures *(187)*, it is more useful for the producer to prevent contamination of fresh produce during production versus sanitizing already-contaminated produce *(187)*. In fact, this is the first principle of the FDA guidelines for minimizing microbial hazards on fresh produce *(81)*. Therefore, practices like Good Agriculture Practices (GAP) and Good Manufacturing Practices (GMP) should be followed to prevent contamination of fresh produce by pathogenic microorganisms. Research has indicated that pathogenic microorganisms can contaminate fresh fruits and vegetables before or after the commodity leaves the production facility *(26)*. Pathogenic bacteria are generally responsible for foodborne

outbreaks linked to produce *(26)*. For instance, *Salmonella*, a common pathogenic microorganism that has been linked to fruit and vegetable outbreaks, may contaminate fresh produce in the pre-harvest or post-harvest stage *(131)*. Irrigation water, worker handling, and contact with soil are other venues by which *Salmonella* can occur on fresh produce *(205)*. There are numerous foodborne illnesses causing human gastroenteritis that have been associated with the consumption of contaminated fresh vegetables and, to a smaller degree, fruit *(26)*. In the U.S., *Salmonella* and *E. coli* O157:H7 have been the two most common bacterial agents responsible for produce-related outbreaks over the past 10 years *(157)*. Based upon ranking criteria established by FAO and WHO, leafy greens and herbs were found to be the greatest concern among fruits and vegetables in terms of microbiological hazards *(81)*. The benefit of leafy green vegetables is they contain a high content of nutrients, such as vitamin K, and minerals such as calcium and potassium. The positive benefits of leafy greens like lettuce, sprouts, spinach, and endive, however, have been counter-balanced by their role as a vehicle of transmission for several foodborne pathogens *(141)*.

Sprouts and microgreens are two vegetable commodities that have a high consumer demand due to their flavor and nutritional significance. Sprouts have been in the news for years while microgreens have just recently gained attention. Both sprouts and microgreens are produced and harvested in an indoor facility with a controlled environment and seldom in an open field production *(234)*. Microgreens can be grown using a traditional planting method, which includes soil or germination mixture, or hydroponically. Sprouts can be produced in any type of container that will allow the

constant watering and adequate temperature and humidity conditions. Sprouts and microgreens share similar potential health benefits; however, they are completely different food commodities. One characteristic of sprouts is that they contain the germinated seeds (seed, root, stem, and undeveloped leaves) when consumed; in contrast, only the stems and leaves of microgreens are intended for consumption *(144)*. There has been a misperception in determining whether there is truly a difference between sprouts and microgreens. Sprouts have been repeatedly connected to outbreaks of foodborne illness. In 2014, the CDC reported an outbreak of *Salmonella* Enteritidis in bean sprouts. Another outbreak in 2016 involving alfalfa sprouts contaminated with *Salmonella* Abony. This outbreak occurred in nine states infecting 36 individuals with 7 hospitalized *(51)*. An *E. coli* O157:H7 outbreak occurred in several locations in Japan (1996). This outbreak was linked to the consumption of radish sprouts, and involved 10,000 cases, mostly young children *(141)*. Due to the multiple outbreaks related to sprouts, the Food and Drug Administration (FDA) continues to regulate any business that seeks to produce sprouts. Currently, the FDA Produce Safety Rule requires sprouts producers to use seeds that have been treated using a scientifically valid approach to reduce microorganisms or rely on prior seed treatment placed by a grower, distributor, or supplier with a Certificate of Conformance documentation *(83)*. While sprouts have become regulated by many national and international standards for their production and distribution, there is a lack of knowledge relating to the safety of microgreens *(234)*.

Due to physical similarities between sprouts and microgreens, and a scarcity of research on the latter, microgreens are often perceived by the public as being in the same

food category as sprouts *(144)*. The assumption is that, since microgreens are grown from the same seed as sprouts, they may pose a similar food safety risk*;* however, there have been no reported outbreaks linked to microgreens to date.

Aim and Significance

The aim of this study is to evaluate major factors that potentially affect the safety of microgreens, such as time of harvesting, harvest practices, and plant type. The significance of this study could clarify the knowledge gap associated with factors involved in the reduction of bacterial pathogens in plants and to whether this decline could label the food product as safe.

Hypothesis

According to several reports, the conditions in which sprouts are grown (high humidity, high temperature, and constant water availability) result in the growth of microorganisms, including pathogenic bacteria if present. Therefore, sprouts produced from pathogen-contaminated seed are expected to contain high concentrations of pathogenic bacteria. In the case of microgreens, whose growing conditions do not consist of high humidity and constant water soaking, the level of pathogen growth may not be as significant. With microgreens, the pathogenic bacteria present on the seed coat should not potentially transfer to the microgreen plant because seed coat will fall off or remain in the soil once the microgreen is fully developed. Therefore, the microgreen edible portions should contain lower levels of pathogenic bacteria compared to non-harvested plant parts such as the seed coat and lower stem.

Objectives

1. Compare the growth and behavior of foodborne bacterial pathogens represented by *Salmonella* serotypes Typhimurium, Saintpaul and Agona, and Shiga toxin-producing *E. coli* (STEC) serotypes O104:H4, O111:H1, O157:H7 and O157:H7 K3999 in sprouts and in microgreens grown from contaminated seed.
2. Determine if the crop variety has an effect on the growth and survival of a cocktail of *Salmonella* and STEC.
3. Determine if the production process and harvest period have an effect on the growth and survival of *Salmonella* and STEC.
4. Determine if inedible portions (lower hypocotyl and seed coats) of microgreens are more populated with the target organism (*Salmonella* Poona) than the edible portions (leaves and middle hypocotyl) using confocal laser-scanning microscopy.

CHAPTER II

LITERATURE REVIEW

Salmonella

Background

Salmonella are Gram-negative facultative anaerobic bacteria belonging to the *Enterobactericae* family. The genus *Salmonella* is named after Daniel Elmer Salmon (1850 to 1914), who first isolated the bacterium in 1885 *(140)*. *Salmonella* consists of multiple species of disease-causing organisms commonly found in the gastrointestinal tract of warm-blooded animals *(110)*. As an intracellular pathogen, *Salmonella* can cause two major types of infections in humans, S*almonella* Typhi infection (typhoid fever) and non-typhi *Salmonella* (gastroenteritis) infection *(233)*. *Salmonella* Typhi is highly adapted to the human host and uses it as its main reservoir, while non-typhoidal *Salmonella* has a broad range of host specificity *(90)*. Non-typhoidal *Salmonella* can be classified into two well-known species, namely *S. enterica* and *S. bongori*. As of 2004, more than 2,500 serotypes have been identified, mostly belonging to *S. enterica*. Today, *Salmonella enterica* is recognized as one of the leading causes of foodborne diseases *(233)*. *Salmonella* contamination can occur in different food commodities such as meat and poultry products, as well as fruits and vegetables, leading to gastroenteritis in humans *(31)*. According to Giannella R. *(93)*, two known clinical forms of *Salmonella* infection arise after ingesting contaminated food: gastroenteritis and enteric fever. After ingestion of the contaminated food, the illnesses caused by *Salmonella* serotypes can be divided into two

classifications, those that remain localized in the gastrointestinal tract and those that are connected to the systematic dissemination of bacteria *(164)*. The localized infections are commonly associated with the consumption of food contaminated with the bacterial pathogens *(164)*.

Bacteriology

Salmonella is a non-sporeforming bacillus, motile by peritrichous flagella and sized at 2 to 5 µm long and 1.5 µm wide *(140)*. The majority of *Salmonella* strains are hydrogen sulfite producers and are catalase positive and oxidase negative *(140)*. According to Grimont et al *(101)*, over 2,500 serotypes exist and can be identified serologically based upon antigenic properties of their lipopolysaccharide (LPS), sugar (O-antigen), and protein (H-antigen) structure components. The H-antigen, also known as the flagellar antigen, is heat-labile and may occur in two forms, phase 1 and phase 2. Some *Salmonella* strains may produce one (monophasic) or two (diphasic) flagella with different antigen specificity *(156)*. The O-antigen takes place on the surface of the outer membrane (OM) and is identified by a certain sugar arrangement on the cell surface *(156)*.

The bacterial cell envelope of *Salmonella* consists of an OM, peptidoglycan, cytoplasmic membrane, and outer sections (the flagella) *(195)*. The OM, found in only Gram-negative bacteria, is located outside the peptidoglycan membrane, and includes two types of lipids, LPSs and phospholipids, as well as a set of characteristic proteins *(162)*. The phospholipid component of the OM contains a minor augmentation in phosphatidylethanolamine similar to the cytoplasmic membrane *(167)*. The LPS; however, is located on the outer portions of the OM and is known as an endotoxin. It is

composed of three parts: the proximal, hydrophobic lipid A region; the hydrophilic O-antigen region; and the region connecting the two, the core oligosaccharide region *(162)*. The LPS may also be an important factor in determining the organism's virulence *(93)*. The next component of the cell envelope is the cytoplasmic membrane. The cytoplasmic membrane is a phospholipid bilayer located between the environment and the cytoplasm with key responsibilities consisting of controlling the movement of nutrients and metabolic products in and out the cell *(195)*. Lastly, the flagella of *Salmonella* are present at random points around the outer surface of the cell in an arrangement known as peritrichous flagella. The numbers of *Salmonella* flagella can range from around 5 to 10 per cell. The overall structure of the *Salmonella* flagellum consists of an extended helical filament, a hook, and a basal body containing a central rod *(162)*.

Metabolic Process

Salmonella can further be classified as chemoorganotrophic heterotroph organisms, possessing both respiratory and fermentative pathways *(67, 156)*. Identification of *Salmonella* through biochemical testing makes it easier to distinguish from other organisms. For example, *Salmonella* are catalase positive, oxidative negative, and can use citrate as a sole carbon source. Thus, they typically produce hydrogen sulfide, decarboxylate ornithine and lysine (amino acids) and do not hydrolyze urea *(108)*. In addition, glucose is the major carbon source for *Salmonella*, which is produced from obtainable substrates through the glycolysis cycle and is mainly broken down in the pentose phosphate pathway (PPP) *(67)*. Once glucose is catabolized, the organism can produce acids and gases such as hydrogen sulfide and CO_2. Generally, a test used to

confirm this trait in *Salmonella* is triple sugar iron (TSI) agar, which contain three major substrates: glucose, lactose, and sucrose. *Salmonella* will not utilize the lactose or sucrose in TSI, only using the glucose to produce gas and acid *(156)*.

Water is essential component of cell metabolism and is necessary for the survival and growth of *Salmonella,* as well as all living organisms. Therefore, water activity is a suitable application in predicting the growth of microorganisms in a food system. Water activity is defined as the ratio of vapor pressure of water in a food matrix compared to vapor pressure of pure water (a_w = p/po) *(5)*. Although mostly used for preservation purposes, it also has been applied as a control measure for food safety. *Salmonella* has a minimum a_w requirement of 0.940; anything lower inhibits growth *(92)*; however, cells are capable of surviving in low moisture foods. As the food's a_w decreases limiting moisture availability, the growth of *Salmonella* is reduced. Several foodborne disease outbreaks have been linked to *Salmonella* involving low-water-activity products, such as peanut butter, chocolate, dried milk, fermented meat, and cereal, as well as dry ingredients like black pepper and paprika *(84).*

A study was conducted to determine the survival of several pathogen strains (including *Salmonella*) in dry environments (a_w of 0.2) at 22°C. After an initial reduction in numbers, *Salmonella* counts remained the same for a majority of the time period; however, it took 249 to 351 days to achieve a 1-log reduction *(117)*. Chocolate is another low a_w food product that has been implicated in numerous *Salmonella* foodborne disease outbreaks. D'Aoust J. *(65)* states although *Salmonella* is incapable of proliferating, it was able to survive for long periods in chocolate, due to the certain conditions held under the

low a_w of this product. Therefore, it was hypothesized that the high fat content in chocolate acts as a barrier for *Salmonella* cells against stomach acid, thus, causing it colonize the G.I. tract and eventually cause severe foodborne illness *(64, 65, 99)*. Nut and seed products like pecans, pistachios, and peanuts are other low moisture foods that have been contaminated with *Salmonella.* A study conducted by Uesugi et al. *(210)* determined the long-term survival of *Salmonella* in almonds. Their results showed *Salmonella* survived for over a five-year period from an almond plantation, and from all the isolates obtained *S.* Enteritidis was the main serotype. They concluded the long-term survival of *Salmonella* may have contributed to reoccurring contamination throughout cycle caused by a range of animal host or by wet almonds from the rainfall.

Osmotic pressure is another key component of water involved in the growth and survival of *Salmonella.* The osmotic pressure can be directly related to a_w *(80),* and used as a preservation method. The incidence of solutes, like salt and sugar, in the food system has an influence on the water activity and growth of microorganisms. For instance, high sugar or salt content in the food causes water to be released out of the cell (via osmosis), which causes the cell membrane to disengage from the cell wall. Thus, by reducing turgor pressure and contracting cytoplasmic membrane this causes inhibition of bacterial growth *(197)*. When osmotic pressure increases, *Salmonella* responds by altering the composition of its OM, this can be a process known as osmotic shock. When osmotic shock occurs, *Salmonella* responds by triggering mechanisms that can increase its internal osmotic pressure and maintain its cell turgor, which happens by the accumulation of metabolic solutes into the cytoplasm *(186)*. For instance, according to Sutherland et al. *(198)*, if the

environment becomes limited in potassium (preferred ion for cell uptake), *Salmonella* can increase uptake of other osmoprotectants, such as proline or trehalose to maintain its internal osmotic pressure and turgor pressure.

pH is another important factor for the growth and survival of *Salmonella.* The optimum pH is between 7.0 and 7.5, with a minimum and maximum of 4 and 9.5, respectively *(92)*. The changes in pH can determine the fate of bacterial growth or survival for instance, as pH decreases, *Salmonella* counts may also decrease. Montville and Matthews *(156)* described the regulation of intercellular pH through mechanisms such as homeostatic response, acid tolerance response (ATR), and acid shock protein synthesis, could prevent denaturing of proteins and permit survival of *Salmonella*. The survival of *Salmonella* under differing acidic conditions has been studied considerably. For example, according to Lee et al. *(135)*, when in the stationary phase, *Salmonella* tends to be relatively resistant to low pH; however, when in the logarithmic phase, *Salmonella* is less resistant to acid and unable to proliferate under moderately low pH (between 4 and 5). With exposure to low pH and the organism growing in the logarithmic phase, there are two distinctive steps of adaptation as described by Rychlik et al. *(186)*. These adaptations are the transient adaptation, which is achieved by exposing *Salmonella* to reasonably low pH for approximately 20 minutes of exposure, followed by a second phase of constant exposure to low pH for approximately 60-minutes.

After a short period of adaption, *Salmonella* can become more acid-resistant *(186)*. This is called ATR *(87, 135)*, and is triggered by a change in pH. In response to low pH, ATR is triggered and *Salmonella* induces proteins and genes, which can be recognized by

a number of methods, including the arbitrary promoter fusion and the selection of genes induced by low-pH promoters. These methods have permitted the identification of genes coding for proteins related to maintenance and cell surface structure (*aas, pbpA*, and *cld*), stress response (*dps* and *rna*), and efflux pump *(emr* and *mar) (214).* In order for *Salmonella* to be able to adapt to low pH environments it must possess numerous regulons regulated by RpoS, Fur, PhoPQ, and OmpR/EnvZ *(186).*

Several studies have evaluated the growth and survival of *Salmonella* under acidic conditions. Golden et al. *(94)* evaluated the survival of *Salmonella* spp. on the inner tissues of cantaloupe, watermelon, and honeydew (pH 5.9). Results indicated *Salmonella* was able to proliferate within the inner tissues of all melons at room temperature (23°C) for 24 h; however, watermelons contained greater counts (1-log) than cantaloupe and honeydew. Another study determined the effect of low pH on the resistance of common foodborne microorganisms including *S.* Typhimurium in forages used in manure. Their study indicated *S.* Typhimurium showed resistance to low pH and some organic acids (citric acid) used in silage *(63).*

Temperature is also another important factor for the growth and survival of all living organisms including *Salmonella*. *Salmonella* is considered a mesophilic organism, the optimum temperature for growth is between 35 and 43°C, with a minimum of 7°C and a maximum of 49°C. Some strains have the capability to survive exceedingly high and low temperatures *(5).* Temperatures below 5°C, usually used for food storage, prevent multiplication of the organism *(92)*; however, low temperatures can cause *Salmonella* to survive in refrigerated products. According to Montville and Matthews *(156),* the

following factors can play a significant role in the survival of *Salmonella* in frozen stored foods: (1) the composition of the freezing environment, (2) the freezing process kinetics, (3) the biological state of *Salmonella,* and (4) serotype-specific responses. During freezing and frozen storage, *Salmonella* may experience rapid death. Slow freezing (0 to -10°C) causes the highest mortality rate, probably because of damage to the cell membrane, while blast freezing usually results in the organism to survive due to less physical damage *(92)*. Studies have demonstrated the presence of *Salmonella* in frozen foods for years, meaning freezing does not guarantee complete elimination of *Salmonella (190)*. For instance, a study was conducted by Dawn et al. *(128)* to determine the survival of *Salmonella* on frozen strawberries. The results indicated when strawberries were stored at 5°C for 7 and 30 days, *Salmonella* counts remained constant, indicating *Salmonella* was capable of survival but not growth.

Temperatures above maximum (49°C) for growth can either destroy *Salmonella* strains or cause them to develop heat resistance under certain conditions. For instance, *Salmonella* heat resistance increases as the a_w of the food decreases. The type of solutes (salt) used to change the a_w can cause *Salmonella* to develop heat resistance *(153, 156)*. Studies have reported that temperatures above 55°C are sufficient to destroy *Salmonella (92);* however, temperatures above 40°C induce heat shock proteins, as described by Rychlik et al *(186)*. These authors defined heat shock as stress inflicted on the cell wall of an organism from the outside and therefore occurs on the outer cytoplasmic membrane.

Infectious Salmonella: Pathology

Salmonella illness in humans and animals depends upon the ability of the organism to survive harsh environments in the gastrointestinal tract before entering the intestinal epithelium, invading the internal organs, and causing systemic infection *(229).* In order for *Salmonella* to become fully pathogenic, it must acquire a selection of properties called virulence factors. Virulence factors include the ability for cell invasion, ability to replicate internally, and the possible elaboration of toxins *(93)*. Upon ingestion of contaminated food, the organism colonizes the ileum and colon of the small and large intestines, followed by attachment to the epithelial tissue, where it is able to proliferate within the epithelium and lymphoid follicles *(216)*. Entry into the epithelial cell may be aided by cytotoxin, which is found on the cell wall of the organism. After entry, *Salmonella* becomes internalized within the epithelial cell by a process known as receptor-mediated endocytosis *(66).* After invasion of the epithelium, *Salmonella* continues to modify its cellular mechanisms to multiply intracellularly and then spread throughout the body; however, with gastroenteritis infections, *Salmonella* remains restricted to the intestines *(93)*. Furthermore, after invasion of the intestines, *Salmonella* induces an acute inflammatory response *(93)*. Through this intrusive progression, *Salmonella* excretes heat-labile enterotoxin, which triggers a net efflux of water and electrolytes into the gastro-intestinal lumen causing damage to the mucosa and thereby leads to foodborne infection, thus resulting in abdominal pain, diarrhea, and fever (*66).*

According to the Foodborne Diseases Active Surveillance Network (FoodNet), non-typhoidal *Salmonella* is estimated to cause around 800,000 to 4,000,000 infections

each year. The cost correlated with *Salmonella* infection is billions of dollars annually, which includes the cost of medical care and loss of productivity *(218)*. Most *Salmonella* infections often cause gastroenteritis, which can range from mild to severe. Typical symptoms associated with *Salmonella* infection are the onset of fever, abdominal pain, diarrhea, and sometimes vomiting which generally last 4 to 7 days *(206)*. *Salmonella* affects the intestines by invading the tissues, causing an inflammatory response. The host responses, importantly, are stimulated promptly after the microorganism is ingested, as indicated by an average incubation period of less than 2 d *(93)*. Gastroenteritis from *Salmonella* can have an incubation period of 8 to 72 hr, depending on the dose of the microorganism consumed *(92)*. Headaches and myalgia are common, but the principal symptom is diarrhea *(127)*. Of those infected by gastroenteritis, almost two-thirds of patients complain of abdominal pain *(93)*.

Salmonella enterica serotype Enteritidis (*S.* Enteritidis) is one of the most common *Salmonella* strains reported in food-related outbreaks. In the U.S. between 1996 and 1999, the prevalence of *S.* Enteritidis in food increased from 5 to 44% *(5)*. According to the CDC, *S.* Enteritidis outbreaks since 2010 have been associated with shell eggs, alfalfa sprouts, pine nuts, and ground beef *(180)*. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is the second-most prevalent strain of *Salmonella* worldwide. *S.* Typhimurium has been linked to outbreaks in ground beef, pork, poultry, and fruits and vegetables *(180)*. Although linked to wide range of food products, both serotypes can use both the animal and human host to cause illness.

Salmonella Contamination of Fresh Vegetables

Each year, around 48 million individuals become ill after consuming food contaminated with pathogens, 128,000 are hospitalized, and 3,000 experience death *(49)*. The CDC estimated that 9.4 million foodborne diseases were caused by 31 known foodborne pathogens, and the majority of all illnesses are caused by seven known pathogens: norovirus, *Salmonella*, *Campylobacte*r, *Listeria monocytogenes*, *E. coli* O157:H7, toxoplasma, and *Clostridium perfringens (58)*. The most common foodborne pathogens reported to cause foodborne illness are norovirus, *Salmonella* and *Clostridium perfringens*. According to reported data on foodborne diseases, *Salmonella* typically leads in the number of cases, hospital visits, premature deaths, and losses of productivity *(22)*. While foodborne pathogens like *E. coli* O157:H7 and *Campylobacter* causing outbreaks have decreased, *Salmonella* has remained constant, making it more of a food safety concern *(37)*.

As reported by the CDC, the number of fruit- and vegetable-related outbreaks doubled twice in two decades, both from 1973 to 1987 and from 1988 to 1992. During these two periods, the agent was unidentified in more 50% of the outbreaks, while for the other half of the outbreaks, *Salmonella* was the predominant etiologic agent *(106)*. Foodborne outbreaks linked to fresh produce, particularly leafy greens, are mostly caused by *S. enterica* and *E. coli* O157:H7 *(22)*.

From the period 2000 to 2008, the sale of vegetables (including potatoes) averaged $17.4 billion *(173)*. However, after 2009, the rate of fruit and vegetable consumption over 5 years per capita decreased by 7% *(174)*. This decline has been driven primarily by due

to behaviors such as not having a dinner side dish. As this decline continues, there are certain commodities that remain the most consumed, such as potatoes, lettuce, green beans, onions, tomatoes, and carrots *(173)*. In addition, the production of vegetables is projected to increase more rapidly than population growth over the next 10 years because of the strong emphasis placed on fitness and nutrition, resulting in increased consumer demand *(174)*.

Produce-borne Salmonella *Illnesses: Prevalence and Outbreaks*

In 1974, temperature abused potato salad containing eggs served at an outdoor barbecue caused approximately 3,400 illnesses. The cause of this outbreak was from a food handler who held the cooked ingredients of the potato salad contaminated with *S.* Newport for up to 16 h at improper temperatures, allowing the organism to multiply. The initial source of the *Salmonella* in the potato salad was undetermined *(156)*. In 1985, one of the worst outbreaks occurred; 6,149 illnesses caused by *S.* Typhimurium were linked to Bluebrook and Hillfarm 2% milk. Cross-contamination of pasteurized milk with raw milk was thought to have been the cause of this outbreak *(56)*. Another large outbreak occurred in South America in 1993, with paprika being the reported contaminated ingredient, found in potato chips distributed from Germany. The source of the outbreak came from one German producer of paprika, which was contaminated with multiple serotypes of *Salmonella* (SaintPaul, Javiana, and Rubislaw) *(156)*. Major foodborne outbreaks linked to *Salmonella* in recent years are of importance because they emphasize the variety of foods and *Salmonella* serotypes that have been associated with human diseases *(156)*. Although *Salmonella* outbreaks are commonly linked to poultry and meat

products, *Salmonella* has also been found to contaminate fruits and vegetables. The Center for Science in the Public Interest (CSPI) *(206)*, reported that *Salmonella* outbreaks linked to fresh produce are increasing, passing the number of chicken-related outbreaks. Between 1990 and 2003, CSPI data analysis estimated over 4,500 outbreaks occurred. Of those, 554 outbreaks were caused by contaminated produce, of which 111 were caused by *Salmonella* alone. CSPI also reported that both fruits and vegetables are commonly linked to *Salmonella* outbreaks, but the most reported commodities are melons, salads, sprouts, tomatoes, lettuce, and other vegetable dishes.

Worldwide, the number of cases and outbreaks linked to fresh produce continues to rise. According to the CDC, between 2006 and 2014, 16 of 68 multistate outbreaks in the U.S. were associated with vegetables *(52)*. Sprouts were implicated as a vehicle for contamination in approximately 38% of those outbreaks. Sprouts have been implicated in numerous foodborne disease outbreaks and have also been labeled as a high-risk food group. Usually contaminated seeds or improper cleaning facilities are often the cause of sprout outbreaks. For instance in 2010, an outbreak investigation identified alfalfa sprouts with water run-off contaminated with *S. enterica* serotype 4,5,12:i. as the vehicle of contamination *(124)*. In 2010, another outbreak caused by *S.* Newport was linked to the consumption of alfalfa sprouts. The growers of the alfalfa sprouts applied practices based on FDA guidelines such as storing seeds under dry conditions and seed treatment with calcium hypochlorite. All standard operating procedures (SOPs) were applied correctly and; therefore, neither violation of food safety practices, nor insanitary condition was

observed according to the growers *(7)*. The *S.* Newport strain was isolated from some seed samples, making the contaminated seed the source of the outbreak.

In the U.S. and worldwide, vegetables like tomatoes and leafy greens also have been associated with a number of well-known foodborne outbreaks *(124)*. In 2004, an outbreak involving 561 infections was caused by *Salmonella*, with the vehicle of concern being contaminated Roma tomatoes. Investigation of this outbreak, led back to one field packaging house from one of the three states involved in the outbreaks as the main supplier of the contaminated tomatoes. The cause of contamination is still under investigation *(206)*. In 2009, an outbreak occurred linking contaminated lettuce to *S.* Typhimurium affecting 10 individuals. However, the cause of this outbreak is still under investigation *(77)*. In 2008, more than 1,400 infected individuals suffered in a multistate outbreak of *S.* Saintpaul *(124)*. The initially suspected source of contamination was tomatoes; however, after epidemiologic investigation by the CDC and FDA, it was concluded that jalapeño and serrano peppers was the actual source of contamination with *S.* Saintpaul *(47)*. Consequently, the failure to identify the peppers as the source of the outbreak resulted in a major economic loss for tomato growers *(154)*. A team of CDC and FDA investigators discovered that 86% of cases were linked to consumption of salsa, guacamole, and raw jalapeño peppers at a Mexican-style restaurant *(22)*. As further examination revealed, the peppers were contaminated before arriving at the restaurant *(154)*. Following GAPs and GMPs would help in the safe production of fresh vegetables. The Food Safety Modernization Act (FSMA) requires FDA science-based standards for the safe production

and harvesting of raw fruits and vegetables (lettuce, tomatoes, and cantaloupe) *(124)*. These standards are useful and essential to reduce the risk to human safety.

The prevalence of *Salmonella* in meat and poultry has been well documented. However, compared to meat and poultry there is minimal reported data on the prevalence of *Salmonella* in fruits and vegetables in the U.S.; those that were reported were limited to pre-and post-harvest practices for ready-to-eat products *(176)*. The prevalence of microorganisms on produce can be done in either qualitative or quantitative form. For instance, a qualitative study was conducted in Texas by Castillo et al. *(42)* to determine the incidence of *Salmonella* and *E. coli* on cantaloupe during pre- and post-harvest practices. The study tested 950 cantaloupes, 140 water samples, and 45 environmental samples from six farms in the U.S. and Mexico. From the samples collected 1.8% were positive for *Salmonella* (fifteen from U.S and nine from Mexico). Water samples collected from four farms (three from the U.S.) were positive for *Salmonella*. From this study, Castillo et al. *(42)* concluded there was no correlation between samples and isolates taken from both locations. Indicating other sources like worker personnel and field environment might also be a factor for product contamination. Another study was conducted by Duffy et al. *(73)* to determine the concentrations of *E. coli* and antibiotic resistant *Salmonella* found in irrigation water, packing equipment and produce (oranges, cantaloupes, and parsley) in Texas during pre-and post-harvest processing. The results showed that of the 1,257 samples collected, 25 *Salmonella* isolates were detected (16 from irrigation, 6 from packing shed, and 3 from washed cantaloupes). *Salmonella*; however, was not detected on

the orange or parsley samples. They concluded from this research that since produce goes through several stages involving different contacts, the source of contamination can vary.

The USDA's Microbiological Data Program (MDP), monitored foodborne pathogens linked to fresh produce consumed in the U.S. The program collected over 12,000 fresh produce samples yearly from distribution centers across the U.S. from 2002 to 2012 and tested for the presence of *Salmonella*, enterotoxigenic *E. coli* (ETEC), and Shiga toxin-producing *E. coli* (STEC). From this study, the commodities chosen for sampling were often consumed and frequently involved in numerous foodborne disease outbreaks *(176)*. Many studies in the U.S. *(122, 207)* have indicated that *Salmonella* presence in fresh produce is normally low (<1%), and the MDP data collection found similar trends. In general, the microbiological laboratories that participated in the MDP program isolated 152 strains of *Salmonella* from fresh produce samples *(176)*. Using polymerase chain reaction (PCR) screening, the commodities found to have the highest prevalence of *Salmonella* were cilantro (0.34%), parsley and spinach (0.29%), hot peppers (0.26%), and sprouts (0.25%) *(176)*. Overall the prevalence of *Salmonella* in vegetables distributed throughout the U.S. was low <1%. From this study, pre-harvest *(170)* and post-harvest stages *(97, 103)* were identified as the potential source of produce contamination. These sources include soil, irrigation water, wildlife, and handling practices *(176)*. Another possible contamination factor for produce commodities grown closer to the ground are lower leaves under surfaces coming in contact with possible contaminated soil or irrigated water *(170, 176)*. The MDP also researched the prevalence of *Salmonella* in imported produce due to the outbreaks caused by *S.* Saintpaul in the U.S. linked to

imported jalapeños *(22)*. MDP tests showed more *Salmonella* strains were collected from domestic produce (123 of 152, or 81%) than from imported produce (22 of 152, or 14%) *(176)*. The second part of the study followed the microbial quality of serrano and jalapeño peppers in Mexico; out of 40 jalapeño peppers tested, one tested positive for *Salmonella* (2.5%). Correspondingly, the majority of imported product samples from Mexico accounted for 20 of 22 (90%) *Salmonella* strains *(176)*.

The MDP data concluded that incidence of *Salmonella* in fresh produce in the U.S. is reasonably low. Even though this may imply that fresh produce may be of good microbiological standing, it does not relate with the increase in *Salmonella* illnesses and outbreaks linked with fresh produce *(176)*. Although the MDP is no longer available, it was a useful resource to monitor incidences of major foodborne pathogens in vegetables.

Other studies like the one conducted by Rude et al. *(183)*, reported the frequency of *Salmonella* (8%) in a collection of vegetable samples obtained from western U.S. market places. Another study reported a 10% prevalence of *Salmonella* found in vegetable samples in New Jersey's market place *(227)*. Ercolani et al. *(75)*, conducted the incidence of *Salmonella* in vegetables, particularly lettuce in Italy. Their results indicated *Salmonella* was found in 68% of the samples. The prevalence of *Salmonella* in vegetable samples obtained from Mexico was also conducted. Of the 100 samples tested from 17 different vegetables, *Salmonella* was isolated from 98% of the samples. Parsley samples had the most *Salmonella* contamination (12%), while cabbage and potato samples had the least (1%) *(175)*. Therefore, from these studies, detecting the prevalence of *Salmonella* in vegetables could help in determining the likely cause of contamination.

Escherichia coli

Background

German microbiologist Theodore Escherich discovered a bacterium in 1884, which he named *Bacterium coli commune*. Dr. Escherich first identified this bacterium in the intestinal tract of infants. It was later named *Escherichia coli* in the 1920s *(24)*. *E. coli* is a Gram-negative, non-sporeforming, facultative anaerobic bacterium belonging to the *Enterobacteriaceae* family. It can be found in the microflora of humans and warm-blooded animals, as well as in the environment. *E. coli* is composed of a diverse group of bacteria. Although there are some harmless strains, there are also pathogenic strains of *E. coli* that can cause illness. The strains that are harmful to humans can cause illness through contaminated food or water. Over 700 serotypes of *E. coli* have been identified, each distinguished by their O- (somatic), H- (flagella), and K- (capsular) antigens *(208)*. The K-antigen is an envelope antigen surrounding the O-antigen that contributes to virulence and prevents phagocytosis from occurring *(159)*. Only the O- and H-antigens are necessary to identify serotypes of *E. coli* linked to diarrheal disease. The O-antigen detects the serogroup of a strain, and the H-antigen determines the serotype *(155)*. Pathogenic *E. coli* can be classified into six pathotypes, each of which is associated with diarrhea *(159)*. The most common group of pathogenic *E. coli*, reported in numerous foodborne outbreaks, is STEC, in particular *E. coli* O157:H7. Ground beef, raw milk, and raw fruits and vegetables are common food commodities connected to *E. coli* O157:H7 outbreaks *(10)*. This particular group of *E. coli* produces the Shiga toxin, which can cause a disorder known as Hemolytic Uremic Syndrome (HUS) that can lead to bloody diarrhea, and

sometimes acute kidney failure. Usually young children and elderly are more susceptible for developing HUS because of their immunocompromised system. Another syndrome related to STEC illness which affects mostly elderly patients is Thrombotic Thrombocytopenic Purpura (TTP). TTP is a rare disorder that occurs when platelets get stuck together forming a blood clot *(204, 177, 163)*. TTP can cause neurological damage as a result of blood clots forming in the brain and fever, and if left untreated it can become fatal. The cause of TTP in STEC patients usually results from a mutation in a ADAMT13 gene that is involved in blood clotting and can eventually lead to destruction of vital organs *(35, 100).* Along with *E. coli* O157:H7, there are other non-O157 strains that will be discussed later on in the chapter that are also capable of producing the Shiga toxin and are just as pathogenic.

Bacteriology

E. coli is a rod shaped bacterium ranging from 1.0 to 1.5 um in width and 2.0 to 6.0 um in length *(162).* The shape of the bacterium is cylindrical and covered in fimbriae. The cell composition of *E. coli*, similar to *Salmonella*, is composed of three principle layers: the OM, the peptidoglycan layer, and the inner or cytoplasmic membrane. Like *Salmonella*, the principal functions for the OM are the transportation of genes, the breakdown of carbohydrates, quorum sensing etc. It also consists of a lipid bilayer located outside the peptidoglycan membrane and contains two types of lipids, phospholipids and an LPS. The LPS plays a crucial role in the barrier layer of the OM and is also responsible for the endotoxic shock associated with septicemia caused by the Gram-negative organism *(195)*. In fact, the sole purpose for the OM is to serve as a protective barrier for *E. coli*

against harsh environments *(129)*. The inner membrane (IM) of the *E. coli* is where energy is produced from breakdown of carbohydrates, lipids, and proteins *(195)*. The major difference between the two membranes in regard to function, is the OM besides being a protective barrier, contains non-specific pores that allow the transport of small molecules through the membrane *(129)*. Lastly, the peptidoglycan is composed of repeating units of disaccharide, which helps establish the rod cell shape of *E. coli*, also protecting the cell from mechanical and osmotic damage *(91)*. Without peptidoglycan, cells lose their distinctive shape *(195)*.

Metabolic Process

E. coli like *Salmonella*, can be classified as chemoorganotrophic heterotroph organisms, possessing both respiratory and fermentative pathways. The primary metabolism for *E. coli* consists of the Embden-Meyerhof-Parnas (EMP) glycolytic pathway, the PPP, the Entner-Doudoroff (ED) pathway, or the TCA cycle *(13, 62)*. *E. coli* proliferates best on simple sugars, such as mono- and disaccharides *(143)*; however, it is unable to proliferate on complex sugars such as starch and glycogen because it lacks necessary enzymes to break down the complex sugars *(76)*. *E. coli* ferments glucose with the production of acid and gas *(162)*. *E. coli* can also utilize amino acids that feed into the TCA cycle in order to gain energy *(62)*.

E. coli can be motile by peritrichous flagella or can be non-motile. From the cell surface, the flagella when present are able to protrude in a peritrichous arrangement *(35)*. The direction of rotation is determined by the movement of the organism. The majority of *E. coli* that are motile use flagella, with rigid structures 20 nm in diameter and 15 to 20

μm long, that project from the cell's surface. *E. coli* uses several flagella positioned alongside the cell body to force cells through mass solutions at a velocity that approaches 20 to 30 μ/sec *(199)*. The swarmer cells of *E. coli* direct their movement when confined in thin layers of fluid *(199).*

Moisture is an essential component for the metabolic process of *E. coli.* As stated previously, water is a suitable function in determining the growth of microorganisms in a food system. As mentioned for *Salmonella*, a food's a_w is defined as the ratio of the vapor pressure of water in a food matrix ($a_w = p/po$) compared to pure water at the same temperature *(5)*. The minimum water activity for *E. coli* is 0.95. Reports have concluded *E. coli* usually does not survive at $a_w < 0.95$; however, in 1993 an outbreak caused by *E. coli* O157:H7 was linked to salad dressing made with mayonnaise ($a_w = 0.93$*).* The outbreak involved 62 individuals who became ill after consuming a seafood salad containing ranch and blue cheese dressing. Investigation of this outbreak traced back mayonnaise as the initial source of the outbreak *(113)*. In 2009, FDA reported samples of a commercial cookie dough ($a_w = 0.80$*)* contaminated with *E. coli* O157:H7, 72 individuals in 30 states became ill after consumption of the contaminated product *(48)*. Since then, questions have been raised regarding the ability of the bacterium to survive in multiple food compounds *(12)*.

pH is another important component affecting the metabolic process of *E. coli.* The microorganism must maintain its intracellular pH, with above or below ideal condition causing the denaturing of intracellular proteins *(24)*. The effect that pH may have on microorganisms could affect the enzymatic functions or the ability to transport nutrients

inside the cell. The optimum pH for *E. coli* growth is 6.0 to 7.0 *(6)*, with a minimum of 4.0 to 4.5 *(156)*. Changes in pH can affect the growth and survival of *E. coli*. For instance, low pH can cause membrane denaturation, which can inhibit growth of *E. coli*. The low pH limit depends on factors like the acidulant used. For example, mineral or inorganic acids like hydrogen chloride are less likely to inhibit *E. coli* compared to organic acids like lactic and acetic acid *(6)*, because organic acids after dissociation from inside the cell, penetrate the cell membrane which causes the release of protons and in return causes the intracellular pH to decrease *(19)*. Peroxyacetic acid was tested in a study to determine its efficacy in preventing *E. coli* O157:H7 on beef carcass surfaces. The peroxyacetic acid had no effect on *E. coli* O157:H7 concentrations; however; lactic acid reduced counts of *E. coli* by 1.9 log CFU/cm^2 *(126)*. Another study was conducted to determine the inactivation of *E. coli* O157:H7 in cucumbers using acetic acid (pH 4.6) and salt. The results indicated acetic acid alone was more effective at destroying *E. coli* on cucumbers compared to in-combination with salt *(136)*. However, in regard to high pH, studies have reported that *E. coli* tends to be more tolerant to low pH rather than high pH. The lack of tolerance to high pH is because many enzymes that play an important role in the metabolic processes for *E. coli* are pH-sensitive *(25)*. When the pH shifts to extreme alkaline conditions, enzymes in *E. coli* become denatured and are prevented from carrying out other functions *(62)*.

E. coli has been shown to tolerate low pH (3.6) environments and to survive under these settings while maintaining proportionality to the degree of contamination *(6)*. After the foodborne outbreak of *E. coli* O157:H7 in apple cider, the FDA suggested that all new

processes for acidified foods include a heating or pasteurization step, especially since *E. coli* O157:H7 has a low infectious dose *(34)*. In fact, because of this outbreak, studies have been conducted to determine the survival and acid tolerance of pathogenic *E. coli* in apple cider. For example, Leslie et al. *(152)* evaluated the survival and acid tolerance of two *E. coli* O157:H7 strains in apple cider. Their results indicated *E. coli* was detectable in apple cider for up to 21 days at 4ºC concluding that *E. coli* is tolerant to low pH. *E. coli* also has the ability to increase its acid tolerance when previously exposed to acidic environments. Montville and Matthews *(156)* described the three systems that are involved in *E. coli* O157:H7 acid tolerance: the acid-induced oxidative system, the acid-induced arginine-dependent system, and the glutamate-dependent system. It is reported that the oxidative system is less efficient in protecting the organism from acid stress than the arginine-dependent and glutamate-dependent systems *(156)*.

Temperature, like pH, plays a role in the activity of enzymes found in *E. coli*. The optimum temperature condition for *E. coli* is 37ºC, with a minimum of 7ºC and a maximum of 46ºC *(6)*. As with pH, temperature variation outside of the normal range can cause enzyme denaturation and loss of cellular functions *(6)*. Some serotypes of *E. coli* have shown the capability to grow and survive in low and high temperatures; however, *E. coli* O157:H7 has been found to be less heat-resistant than other pathogens *(156)*.

Temperatures below the minimum growth range usually cause inhibition of growth *(123)*. However, it has been reported that *E. coli* survives well in chilled and frozen foods. For instance, when *E. coli* was inoculated on ground beef and stored at –20ºC for 9 months, little change was observed in counts. The report observed an initial 1-log reduction

followed by slow decline in cell numbers *(6)*. In a study conducted by Knudsen et al. *(128)*, the survival of *E. coli* O157:H7 on cut and whole frozen strawberries was determined. Results indicated when stored at -5 ºC for 7 days, counts remained constant on cut surfaces, but whole surfaces experienced up to 1-log reduction. However, after 30 days of storage counts of *E. coli* declined by as much as 0.7-2.2-log on both surfaces.

Temperatures above the maximum growth range can cause the organism to experience rapid death because of alterations of niches that occur when exposed to such high temperatures *(165)*, or cause it to produce a heat shock response *(184)*. *E. coli is* capable of surviving above maximum temperature and can progressively adapt to the temperature by increasing its thermal optimal conditions so that it is able to continue to survive in the upper maximum temperature range. This process is referred to as a sliding niche, and *E. coli* tolerates better adaptation to the temperatures in this environment. *E. coli* is also able to alter its genetic information in order to proliferate at its maximum rate while facing a constant temperature change, as long as intrinsic factors such as nutrients and pH are favorable for growth *(165)*.

Diarrheagenic E. coli

E. coli was first considered a foodborne pathogen in 1971, when imported cheeses in 14 states were contaminated with an enteroinvasive strain causing infections in 400 individuals *(119)*. Shortly after 1971, outbreaks began to arise in other countries. The most common group of pathogenic *E. coli* reported in multiple foodborne outbreaks is STEC, in particular *E. coli* O157:H7 *(10)*. Common food sources for contamination are raw or undercooked ground beef products, raw milk, and raw fruits and vegetables. Symptoms of

STEC after consuming some of these contaminated food products include diarrhea, abdominal pain, and HUS *(232)*.

Even though the STEC group has more commonly reported outbreaks, other pathotypes have been involved in frequent outbreaks. Their serogroup outlines are Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), and Enterohaemorrhagic *E. coli* (EHEC) *(61)*. Since the term Enterohaemorrhagic *E. coli* is outdated, this *E. coli* group will be referred to as Shiga toxin-producing *E. coli* (STEC)

Enteropathogenic *E. coli* (EPEC). Approximately over a million deaths occur annually in children under the age of five globally, the second major cause of death is diarrhea *(30)*. EPEC are one of the diarrheagenic *E. coli* groups which can cause severe diarrhea in young children especially infants *(72)*. EPEC is transmitted by the fecal-oral route through contaminated hands or foods (formula milk). The organisms attach to intestinal mucosa, leading to disposition of the host cell *(147)*.

Occurring only twice in the U.S., EPEC caused reoccurring foodborne outbreaks of infant diarrhea. They occurred primarily in daycare centers and pediatric facilities *(209)*. EPEC outbreaks mostly occur in developing countries. In Northern France, EPEC caused two foodborne outbreaks (59 cases) linked to mayonnaise, lettuce, and gherkins *(230)*.

Enteroaggregative *E. coli* (EAEC). First discovered in a child with acute diarrhea in Chile in 1987, EAEC has since been connected with persistent diarrhea in children residing in areas where there is an endemic *(224)*. In the U.S., among adults traveling to

developing countries, EAEC is the second most common bacterial pathogen *(2, 185)*. EAEC includes strains of *E. coli* that do not secrete the enterotoxins liable-toxin (LT) or stable-toxin (ST), but attach to HEp-2 cells in an aggregative attachment pattern *(164)*. According to Debroy et al. *(69)*, EAEC contains a 60-MDa plasmid that is required for the production of fimbriae proteins that are responsible for the distinct aggregative pattern. EAEC strains attach to mucosal epithelium by bundle-forming EAEC fimbriae structures. After attachment, the second stage involves adherence to the thick mucosa with the presence of an aggregating biofilm *(221)*. The third stage involves the release of the toxin, causing damage to the mucosa and intestinal secretion *(1)*. This damage is caused by serotypes of groups O111:H12 *(40, 191)*, O125:H21 *(1)*, and O128:H35 *(1)*.

Enteroinvasive *E. coli* (EIEC). EIEC was first discovered in 1947 among school children, with salmon being the reported vehicle *(119)*. EIEC strains are moderately related to *Shigella* species in that they colonize the epithelial cells and spread to adjoining cells; however, they do not produce the Shiga toxin *(147)*. The strains can be recognized by their ability to invade HeLa cells and are confirmed by an invasive Sereny test (keraconjunctivitis) in guinea pigs *(164)*. EIEC strains have been known to have a preference for the colon, with bloody or watery diarrhea being an outcome *(119)*.

An outbreak in Texas reported in 1994 involved restaurant-associated infections of 370 individuals. The reported vehicle of this outbreak was contaminated guacamole prepared by a local catering company in Houston: however, route of contamination is still undetermined *(96)*. Reported EIEC outbreaks are mostly foodborne or waterborne, although person-to-person contact has been reported with these strains *(209)*.

Enterotoxigenic *E. coli* (ETEC). ETEC was first discovered in the 1970s by outbreaks linked to contaminated food and water *(181, 209).* ETEC strains, similar to *Vibrio cholera*, attach to small intestinal mucosa by surface fimbria (type 1 pili) and produce symptoms not by invading the mucosa, but by developing one or two enterotoxins (heat-labile toxin LT and heat-stable toxin ST) *(147).* The LT toxin is a protein with a molecular weight of about 91 kDa *(119),* with similar properties to cholera toxin (CT). The LT toxin is destroyed at 60ºC for 30 minutes, while ST can survive at 100ºC for 15 min *(119).* The invasion of the intestinal mucosa by the enterotoxins causes diarrhea *(147).* Unlike EPEC, these strains cause diarrhea in both children and adults. ETEC strains are most recognized for causing traveler's diarrhea in the U.S. *(119).*

Foods that are usually involved in ETEC outbreaks are cheese, curried turkey, mayonnaise, crabmeat cocktail, and drinking water *(209).* Two outbreaks of ETEC have occurred: one in Rhode Island involving 47 passengers of a flight from Charlotte, NC to Providence, RI, and the other involving 121 individuals attending a buffet dinner at a hotel lodge in New Hampshire. *E. coli* O6:NM was isolated from individuals in both outbreaks. The reported vehicle for these two outbreaks were carrots that were grown in the same state, and used in salads that were distributed between these two states *(43).*

Shiga Toxin-Producing *E. coli* (STEC). Many strains of *E. coli* belong to the STEC group, with *E. coli* O157:H7 being the predominant foodborne pathogen *(147). E. coli* O157:H7 was first recognized as a human pathogen in 1982 when consumption of contaminated undercooked ground beef caused two outbreaks of hemorrhagic colitis *(178).* Other non-O157 members of this group are O26, O45, O103, O111, O121, and

O145. STEC can cause HUS, mainly in young children and adults. HUS is a severe condition that causes bloody diarrhea and can result in kidney failure *(59)*. STEC strains are similar to EPEC, except they produce toxins often referred to as Shiga-like toxin (verotoxin, verocytotoxin) and two prototypes, Stx1 and Stx2. They both consist of a single active A subunit and multiple B subunits, and in some STEC strains, genes for Stx1 and Stx2 are encrypted by bacteriophages *(119)*. Jay et al. *(119)* describes after the toxin binds, followed by internalization, the A subunit binds and releases an adenine residue, which inhibits protein synthesis while, the B subunit forms a pentamer in link with a single A subunit and therefore accounts for the binding of the toxin to the glycolipid receptors.

Cattle are often considered the main reservoir for *E. coli* O157:H7 *(18)*. Many outbreaks caused by *E. coli* O157:H7 have been linked to the consumption of undercooked ground beef *(178)*. Even though ground beef has been the common commodity linked to *E. coli* O157:H7 outbreaks, other foods have also been involved in outbreaks linked to *E. coli* O157:H7, such as raw milk, yogurt, lettuce, unpasteurized apple cider/juice, cantaloupe, radish sprouts, and alfalfa sprouts *(148)*. Sprouts have been recently added to the spectrum of foods as a vehicle of *E. coli* O157:H7 *(147)*. There was a large outbreak involving radish sprouts in Japan in 1996 and 1997 *(200)*. In addition, two other outbreaks involving alfalfa sprouts caused STEC infections in Michigan and Virginia *(44)*.

Shiga Toxin-Producing *E. coli* Contamination of Fresh Vegetables

As previously mentioned, according to CDC (2014) one in six Americans becomes ill due to contaminated food, 128,000 are hospitalized, and over 3,000 die. The agents that cause the most outbreaks are bacteria, viruses, and parasites *(49)*. Determining the

economic loss caused by these foodborne pathogens can be difficult because not all incidences are reported. According to ERS the annual economic cost of STEC, *E. coli* O157 in particular, estimated around $405 million for O157 and $154 million for non-O157 *(189, 212)*. The cost includes medical costs like kidney dialysis and transplants, time cost due to loss of work, and cost of premature death *(212)*.

Numerous studies have shown cattle are considered the main reservoir for STEC organisms *(31, 53, 121)*. However, over the past 30 years, the rise of foodborne disease outbreaks linked to fresh fruits and vegetables have started to become more apparent, especially those linked to pathogenic *E. coli (145).* For instance, *E. coli* O157:H7 continues to be the leading serotype in the STEC group to cause outbreaks linked to fruits and vegetables. The CDC estimated between 1973 – 2012, STEC was the second leading cause of foodborne disease outbreaks linked to leafy vegetables, of which the O157 group accounted for majority *(46, 107)*. The spinach outbreak in 2006 caused by the O157 group, was one of the deadliest foodborne outbreaks to occur in the U.S. attributed to leafy vegetables *(228)*. However, other non-O157 serotypes should be considered equally as important in regard to the safety of produce since they have also been implicated in produce outbreaks. For instance, O111, O121 and O145 have been linked to lettuce outbreaks and O45 has been linked to clover and alfalfa sprout outbreaks *(79).*

Produce-borne STEC *Illness: Prevalence and Outbreaks*

According to the CDC, produce-related outbreaks were first reported in 1991 and are still a significant problem in 2017 *(45)*. They account for approximately one third of total foodborne outbreaks occurring the U.S. Montville and Matthews *(156)*, describes

from 1982 to 2002, there were 350 outbreaks of *E. coli* O157:H7 reported in 49 states in the U.S. *(156)*. Even though the number of outbreaks continued to increase, the median outbreak size decreased between 1982 to 2002.

Multiple outbreaks in the U.S. over the years have been associated with fresh fruits and vegetables such as lettuce, apple cider, unpasteurized apple juice, and alfalfa sprouts contaminated with STEC *(145)*. As described by Sivpalasingam et al. *(196)*, there have been 25 lettuce-associated outbreaks causing over 2,000 illnesses, 181 hospitalizations, and six deaths. The outbreaks were caused by 17 known pathogens including *E. coli* O157:H7, which was reported in three states (New York, Connecticut, and Illinois). With the number of STEC outbreaks linked to leafy vegetables continuing to increase, STEC has been considered the leading cause of leafy vegetable outbreaks with confirmed etiology *(107)*. STEC caused over two-thirds of multistate outbreaks, with leafy vegetables being responsible for 45% of hospitalizations and almost half of the deaths *(107)*. As previously mentioned, in 2006 a STEC outbreak (O157:H7) involving contaminated spinach products became known as the deadliest U.S. foodborne outbreak attributed to leafy vegetables ever reported, causing almost 200 confirmed cases, 100 hospitalizations, and five deaths *(228)*.

According to the CDC, in 2015 an outbreak of *E. coli* O157 occurred causing 19 individuals to become ill after consuming salads purchased from Costco *(50)*. Approximately 5 individuals were hospitalized and 2 developed HUS. The initial source of the outbreak was rotisserie chicken found in salads; however, after further investigation, vegetables in the salad were considered the main source of the outbreak. According to

FDA, celery mix and onions found in the salad were contaminated with *E. coli* O157, and traced back to a local farm in California *(84)*. Another outbreak linking *E. coli to* vegetables occurred in 2016 at several Chipotle restaurants. According to the FDA, 55 individuals became ill after consuming food contaminated with Shiga toxin-producing *E. coli* O26 *(85)*. The source of the outbreak has been traced back to vegetables sold at the restaurants; however, further investigations are still being determined.

Sprouts are one commodity that have been linked to well-recognized STEC infections. Radish sprouts, in particular, were reported in a Japan outbreak in 1996, including the massive Sakai city outbreak *(160)*. The outbreak in Japan involved 10,000 cases of *E. coli* O157:H7 infection, most of which were reported in school-aged children *(160, 161)*. Another outbreak occurred in 2016, where state, federal, and local health officials investigated an outbreak linked to the consumption of alfalfa sprouts contaminated by *E. coli* O157:H7 *(57)*. The producer of the alfalfa sprouts was the Jack & the Green Sprouts Corporation located in Wisconsin. Eleven individuals became infected as early as January 17 to February 17, 2016, in two states, Minnesota and Wisconsin, only two became hospitalized, and none developed HUS *(57)*. Sprouts seem to pose more of a food safety hazard since pathogens present in low numbers may multiply during sprouting *(44)*.

Since first being recognized in 1982, STEC infections have continued to be reported in more than 30 countries *(156)*. Yearly prevalence rates of *E. coli* O157:H7 have been reported in regions of Canada, Scotland, and the U.S. *(100)* at 8+ per 100,000 individuals *(145)*. Although the prevalence of STEC in ground beef has been studied

extensively, very few studies; however, have determined the prevalence of STEC in vegetables. Each year, more illnesses are linked to leafy vegetables (22%) than any other food commodity *(169)*. Multiple studies have indicated leafy greens to be the reported vehicle for half of norovirus outbreaks, with the second most reported being *E. coli* O157:H7 *(169)*. Since leafy vegetables are one of the most frequently associated commodities in foodborne outbreaks in the U.S. *(81)*, few studies have determined whether the prevalence of STEC would be either high or low.

MDP analysis indicated STEC serotypes to be isolated from different types of produce in the U.S., primarily leafy vegetables. The MDP analysis from a 10 year testing period, with 2,200 samples tested annually, showed STEC was present in spinach (0.5 to 0.6%), cilantro (0.3%), and lettuce (0.04 to 0.18%) samples *(79)*. According to MDP, the incidence rates achieved here do not represent the overall trend of STEC found in lettuce because in previous years the prevalence of STEC was higher. The surveillance from this study suggested that STEC prevalence may change throughout the years depending on factors such as location and time. Also in response to the 2006 spinach outbreak, MDP incorporated spinach into the testing plan. The incidence of STEC isolations in spinach remained steady from 2009 to 2011; however, in 2012 the prevalence rates of STEC in spinach increased by as much as 1%. For verification purposes, MDP serotyped all STEC strains by the *E. coli* Reference Laboratory at Pennsylvania State University. Only half of the strains could be identified, and those that were identified were recognized as O157:H7, O26:H11, O121:H19, and O113:H21, as well as O165:H25 and O91:H21.

Another study led by Saeed et al. *(111)* in the Middle East, to determine the prevalence of *E. coli* O157:H7 in different vegetables collected from different market places around the country. Two hundred samples were collected, and of those samples generic *E. coli* was isolated in 19% of the samples with the highest being parsley (90%) and the lowest being tomatoes; however, *E. coli* O157:H7 was not found in any of the samples. In Canada surveillance of three major pathogens including *E. coli* O157:H7 was investigated in six vegetable commodities (leafy greens, tomatoes, leafy herbs, berries, green onions and cantaloupes) collected from several marketplaces across the country. Their results indicated out of the 23,805 samples tested, the prevalence of *E. coli* O157:H7 was not detected in any of six commodities, but generic *E. coli* was detected in tomatoes and berries samples (<1%) *(71)*. Their findings revealed contamination of fresh fruits and vegetables with bacterial pathogens, including *E. coli* O157:H7, is low in the Canadian marketplace, thereby implying food safety practices carried out by farmers and distributors are generally good.

Microgreens

Background

Fresh produce has increased in popularity as a healthy choice for a well-fit diet due to high nutritional value and intense flavor. Produce can be consumed either slightly processed or raw. These reasons alone have resulted in an increase in the demand for these products, especially among health-conscious consumers. Within the produce group, there is the vegetable group, described by stem, leaves, and shoots from various leafy plants. Within this group are leafy greens. Leafy green vegetables contain several micronutrients,

such as vitamin K and minerals like calcium and potassium that are supplied to the diet. Alongside their positive impacts associated with nutritional value, vegetables such as lettuce, sprouts, spinach, and endive have been implicated as vehicles of transmission of several foodborne pathogens like *E. coli*, *Salmonella*, and *L. monocytogenes (82).* Since vegetables are often grown in open fields and in close proximity to the ground, they have a relatively higher risk of contamination with bacterial pathogens compared to other food commodities. Current research has indicated that pathogenic organisms can contaminate fresh fruits or vegetables before or after the commodity leaves the production grounds *(26).*

The vegetable group has two food commodities that share similar health benefits, high demand among consumers, and production at early stages of development. They are sprouts and microgreens. Normally, both sprouts and microgreens are produced in an indoor facility which allows for a controlled environment compared to open field production for pre- and post- harvest practices *(235).* Although both sprouts and microgreens share similar health benefits, they are two different food commodities. Microgreen growers from Good Water Farms stated, "microgreens could be easily confused with sprouts, but they aren't the same thing" *(16).* Certain characteristics that differentiate the two is sprouts, for instance, contain the germinated seeds (seed, root, stem, and undeveloped leaves) when consumed, while microgreens only contain the stem and leaf when consumed. Sprouts are harvested at an early stage of development (5 days), while microgreens are harvested later (14 days). Microgreens also have the option to be

grown hydroponically or using a traditional planting method, which includes soil/germination mixture.

It is well known that sprouts can represent a high food safety concern because the conditions in which they are grown (temperature, time, humidity, and nutrient availability) are ideal for the proliferation of foodborne pathogens *(83, 86, 235).* In a study conducted by FDA researchers *(51),* increased temperature and moisture were determined to be contributing factors to the growth of *Salmonella* on sprouts. Thereby stating, as temperature increases, the growth rate of *Salmonella* is higher. According to FDA (1998), the reason sprouts represent a major problem is because foodborne pathogens that may be present at very low levels on the seeds can multiply to high levels during sprouting *(83).* Due to numerous sprout-related outbreaks, FDA recommends using the Produce Safety Rule guidelines for sprout producers, which require seeds be treated using a valid scientific approach to reduce microorganisms or to rely on prior seed treatment placed by grower, distributor, or supplier with a Certificate of Conformance *(83)*.

As previously stated, microgreens are tender vegetable plants harvested usually at the cotyledon or the first true leaf stage *(9, 125).* They are often confused with sprouts because they are also harvested at an early stage of development. While microgreens are grown in soil and develop a stem and one or two pairs of leaves, sprouts are harvested soon after seeds are germinated and are produced entirely in water *(144).* Although microgreens have not been around for long, they are popular in salads among consumers due to their high nutritious components, taste, and appearance *(23),* and they have the potential to gain a substantial percentage of a $500 million sprout market *(36).* Some popular varieties of

microgreens include arugula, kale, basil, cilantro, and broccoli, among others shown in Table 1. Nutritional studies have indicated that microgreens are a suitable source of vitamins and phytonutrients such as carotenoids *(225, 226)*. For instance, the Brassica family (cabbage, broccoli, and radish) is well recognized for containing high amounts of glucosinolates, as well as vitamins, minerals, and carotenoids. Warner J. *(225)* discovered that red cabbage microgreens contain 40 times more vitamin E and 6 times more vitamin C, while cilantro microgreens contain 3 times more beta-carotene compared to mature vegetables. The amount of nutrients in microgreens is essential for skin, eyes, and overall health *(225)*.

Through the years, the production of microgreens has developed from small local operations to large-scale operations. The young, edible greens can be grown using two current production systems: in soil-based media or in non-soil-based media, like hydroponic pads. They are usually harvested 2.5 cm above the soil surface. Although the soil-based media method is simple and convenient, the hydroponic system is a newer and popular method. Hydroponic pads use an absorbent padding, typically made from biodegradable wood fibers. The advantage that producers have when using hydroponic padding is elimination of soil setup and less labor exertion. However, when dealing with food safety, the hydroponic production system would seem to cause a higher food safety risk versus using a soil substitute because hydroponic conditions create a more favorable environment for bacterial growth due to the presence of more water. A study was conducted at the University of Maryland comparing a hydroponic system to the traditional method using radish microgreens contaminated with *E. coli* O157:H7 to determine which

Table 1. Variety of Microgreens

Market Name	**Family**	**Genus and Species**	**Average Days to Harvest (After Germination)**
Amaranth	Amaranthaceae	*Amaranthus cruentus*	8 to 12 days
Arugula	Brassicaceae	*Eruca sativa*	5 to 7 days
Basil	Lamiaceae	*Ocimum basilicum*	14 to 21 days
Beet	Chenopodiaceae	*Beta vulgaris*	8 to 12 days
Broccoli	Brassicaceae	*Brassica oleracea L. var. italica*	5 to 7 days
Celery	Apiaceae	*Apium graveolens*	14 to 17 days
Chard	Chenopodiaceae	*Beta vulgaris*	8 to 12 days
China Rose Radish	Brassicaceae	*Raphanus sativus*	10 to 14 days
Cilantro	Apiaceae	*Coriandrum sativum*	
Clover	Trifolium	*Trifolium sp.paratense*	8 to 12 days
Endive	Asteraceae	*Cichorium endiva*	8 to 12 days
Mustard	Brassicaceae	*Brassica juncea*	6 to 10 days
Red Cabbage	Brassicaceae	*Brassica oleracea L. var. capitata*	4 to 7 days
Purple Cabbage	Brassicaceae	*Brassica oleracea*	4 to 7 days
Pac Choi	Brassicaceae	*Brassica rapa*	7 to 10 days
Radish	Brassicaceae	*Raphanus sativus*	6 to 8 days

method would allow greater growth. The results of the study indicated that *E. coli* O157:H7 proliferates more in a hydroponic system compared to a soil system, most likely due to the fact that the hydroponic system provides a more favorable environment for bacterial growth through increased moisture and water availability *(234)*.

The time from seed to harvest of microgreens differs between plant species, but they are typically harvested between 2 and 4 weeks *(9)* and are usually cut 1 inch above the soil surface *(11)*. Researchers Kasier and Ernst *(125)* stated that microgreens are harvested at the first true leaf stage with roots left behind and having only stem and leafs attached. Mickens J. *(150)* stated that the harvesting of microgreens occurs between 7 and 20 days after planting, either at the cotyledon stage or the first pair of true leaves stage. Grant Z. *(98)* from the University of Illinois also stated that the harvesting of microgreens can be done at the cotyledon or true leaf stage.

When the harvesting period approaches, appropriate equipment is needed to ensure a harvest of maximum plant matter. With microgreens, scissors are commonly used. Typically for lettuce, plants are cut about 1 in. (2.5 cm) above the soil surface *(179)*. In the case of microgreens, a production trial was performed by a team of researchers, in which they cut microgreens as close to the soil surface as possible *(8)*. Their results indicated no major quality issues were observed when cutting close to soil surface. In regard to food safety, this practice may cause higher contamination levels since the plant is harvested so close to soil surface where microorganisms could reside.

Harvest period and production practices may a play a role in the growth of bacterial pathogens found on vegetable plants. There have been many studies attempting to explain

pre- and post-harvest factors that influence interactions between *E. coli* O157:H7 and leafy greens *(70)*. Researchers discovered that under laboratory settings, during the extended harvest period *E. coli* O157:H7 was able to survive on different locations of the plants *(223)*. This may be the same circumstances for microgreens, although there is not enough published research to determine with evidence if harvest period and production practices have an influence on pathogen growth with microgreens.

Sources of Contamination

Foodborne outbreaks have become associated with the consumption of fruits and vegetables *(138)*, and vegetables were reported in 2008 by the FDA and World Health Organization (WHO) as the highest concern in terms of produce safety *(54)*. In many vegetable-related outbreaks involving sprouts, the seed is usually the initial source of contamination. These commodities are often produced in an open field where they are more susceptible to contamination *(54)*. Therefore, GAPs and GMPs should be put in place to reduce the risk of product contamination during production and processing *(95)*. Factors that contribute to vegetable contamination are the environment, processing, and packaging, i.e., pre-harvest and post-harvest. Although not connected to any foodborne outbreaks, microgreens can be a source of contamination of bacterial pathogens.

Pre-Harvest. Throughout the production line, bacterial pathogens may have the opportunity to contaminate vegetables at any step *(38)*. Possible pre-harvest contamination sources like soil, feces, irrigation water, dust, insects, wild and domestic animals, and worker handling are all likely sources *(26)*. Untreated manure can be a source of contamination for vegetables, especially through the use of fertilizer or irrigation water. It

has been well documented that *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* can be found in animal feces *(38)*. Additionally, the spread of *E. coli* O157:H7 from manure to contaminated soil, water, and to the vegetable plants has recently been reported *(220)*. Therefore, if manure is not properly composted, contamination of vegetables is likely to occur. Other studies have reported that the introduction of pathogens can occur through the use of water. Therefore, irrigation wells should be maintained properly and all irrigation sources should be examined for human pathogens. Vernozy-Rozand et al. *(217)* determined the presence of *E. coli* STEC in manure, compost, and slurries. Results indicated thru PCR testing that 24% of the samples were positive for Stx2, 33% for Stx1 and 19% for the *eae* gene. One strain from each of the serotypes O157, O26, and O55 was also detected *(217)*.

Since *Salmonella* can be found in sewage water that can be used to irrigate fresh produce, and since there have been foodborne outbreaks in recent years linked to multidrug-resistant *Salmonella* serotypes, emphasis has been put on the need to eliminate all *Salmonella* from irrigation water *(28)*. Duffy et al. *(73)* measured the incidence involved in contamination of produce with bacterial pathogens. Chosen samples of food sources (cantaloupe, oranges, parsley) and environmental samples such as soil, irrigation water and equipment were collected to determine *Salmonella* and *E. coli* prevalence. Of the samples collected, *E. coli* was found in all commodity and environmental sources, while *Salmonella* isolates were found in irrigation water, packing equipment, and 3 of the washed cantaloupes. The study showed *Salmonella* was isolated from 160 out of 170 (94%) irrigation waters examined. Therefore, since *Salmonella* has been reported in

waters used for the irrigation of produce, it is crucial to test the microbiological quality of water in order to prevent contamination of produce.

Apart from farm animals coming in contact with produce, wild animals like birds and reptiles can be a vehicle for foodborne pathogen *(28, 38)*. A study was conducted by a team of researchers who collected fecal samples from wild birds to determine the presence of *E. coli* O157:H7 at a metropolitan landfill in the UK *(222)*. The results concluded that 0.9 to 2.9% of the collected samples were positive for verocytotoxin-producing *E. coli* VTEC.

In general, soil, manure, irrigation water, and wildlife are pathways for the introduction of foodborne pathogens to fresh produce. Since fruits and vegetables are intended to be eaten raw, emphasis is put on preventing the introduction of these pathogens. Therefore, prevention of pre-harvest contamination is a fundamental part of a systematic method focused on ensuring microbiologically safe fruits and vegetables for human consumption *(28)*.

Post-Harvest. Post-harvest contamination sources include personnel handling, harvest equipment, transport vehicles, water, domestic animals, and processing equipment. Practices should be in place to ensure as little contamination as possible. For instance, according to Buck et al. *(38)* worker hygiene and human waste management should be imposed at production sites as well as rules listed for GAPs should be followed to reduce the risk of a food safety hazard.

Worker management should ensure the use of good agricultural and management practices are being placed to reduce the risk of direct or indirect contact with fecal matter

and fresh produce. FDA (1998) states all employees should follow rules for protecting worker health as established by the U.S. code of federal regulation (CFR) title 21, section 110.10, which describes hygiene practices within the context of GMPs in the processing, packing, or holding of food products *(82)*. Previous outbreaks with fresh produce have usually been connected to fecal matter *(82)*. A study was conducted by Jimenez et al. *(120)* to determine the bidirectional transfers of *S.* Typhimurium to green bell peppers by using gloved versus bare hands. The study revealed that a 1-log reduction was observed on the bare hands with a combination of hand washing and alcohol based hand gel.

Harvest equipment such as knives, storage containers, and equipment designed for harvesting produce can be sources of contamination of the final product. Field trimming and coring of lettuce is a practice commonly used because it reduces shipping expenses and waste disposal *(124)*. Yet, core removal can cause tissue damage, which can increase the risk of microbial contamination thru cut edges *(81)*. Taormina et al. *(203)* reported that knives used for coring can transfer *E. coli* O157:H7 to lettuce heads after direct contact with pathogen-contaminated soil. Machine harvesting has been gradually used due the minimal time it takes to use the machine versus manual labor, but it also can increase the risk for surface contact exposure *(78)*.

The processing operations can provide several opportunities for contamination by cutting, washing, packing, and storage *(124)*. Cutting of vegetables causes the release of exudates (nutrients) that can assist in the growth of foodborne pathogens *(138)*. Another study by Brandt M.T. *(33)* showed that tissue damage of shredded lettuce resulted in an increase of *E. coli* O157:H7. Washing vegetables removes soil from the produce surface

and can extend the shelf-life by removing some microorganisms; however, it can also be a vehicle for contamination on fresh vegetables. Washing of vegetables with water of low microbial quality can serve as a vehicle for dispersion of microorganism *(109)*. Perez-Rodriquez et al. *(172)* also conducted a study for wash water evaluation, where they observed *S.* Enteritidis was spread to fresh cut vegetables during processing by the wash water.

There will always be a need for continued methods to develop best practices for production and processing of fresh vegetables to reduce the risk of contamination of foodborne pathogens *(124)*. For both pre-harvest and post-harvest practices, it is best to continue to follow GAPs and GMPs to reduce microbial contamination.

Methods to Eliminate Foodborne Pathogens

Although microgreens and other vegetables do not contain a kill step to eliminate pathogens, the need for an effective antimicrobial is crucial to ensure the product remains safe for human consumption. Washing has remained a widely used technique, which can extend the shelf-life of fresh vegetables by reducing surface microorganism *(38)*; however, not all microorganism are eliminated. Although only a percentage of pathogens may be removed by washing, and the use of a disinfectant can be an additional measure to reduce those pathogens that still remain on the surface *(32)*. The use of a sanitizer and its effectiveness depends on the pathogen. For instance, *L. monocytogenes* has been known to be more resistant to chlorine compared to *Salmonella* and *E. coli* O157:H7 *(27)*. The lack of the sanitizer's effectiveness can also depend on the physical structures and the tissues within the vegetable that can harbor pathogens *(38)*.

As previously stated, in most vegetable-related outbreaks involving sprouts, usually the seed is the initial source of contamination. The use of physical and chemical treatment for elimination of pathogens can be applied to seeds in order to reduce the chance of bacterial contamination. Buck et al. *(38)* described barriers that might prevent this treatment from being effective for the seed: 1) treatment doses that are able to eliminate bacterial pathogens without affecting seed viability; 2) the seed itself may cause the treatment to become less effective; and 3) locations of the bacteria that are protected by the seed. The use of chemical treatments for seed has been reported and includes chlorine compounds (sodium hypochlorite), ethanol, hydrogen peroxide, and ozonated water *(27, 134, 202)*.

Hot water treatment of seed has also been used to eliminate bacterial pathogens *(38)*. This method involves subjecting seeds to temperatures of 55°C for 10 to 15 mins *(102)*. Increased temperatures will eliminate the microorganism found on the seed but the greatest concern with this treatment is the negative effect it will have on seed germination *(38)*. Although hot water treatment has shown some effectiveness, combination with chemical treatment has also shown to reduce microorganism. For example, the combination of heat treatment and chlorine has reduced populations of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds *(118)*. When *Salmonella*-inoculated alfalfa seeds were treated with 100-290 ppm chlorine solution for 5-10 minutes significant reduction was observed ($P<0.05$). Followed by treatment of hot water at 54-47°C caused the reduction of *Salmonella* counts at <1 log CFU/g *(29)*. Although both methods were able to reduce *Salmonella* counts significantly, complete elimination was not accomplished.

Overall, the majority of seed sanitization methods are able to reduce, but do not entirely eliminate, pathogenic bacteria. Pathogen reduction alone is not sufficient to consider the produce as safe because even low concentrations of microorganism pose a substantial health risk *(38)*. Therefore, the need for continued treatment is crucial for the elimination of bacterial pathogens on fresh vegetables.

Confocal Laser-Scanning Microscopy

Background

The confocal imaging microscope is a system that provides different imaging techniques at different resolutions. The major purpose of the confocal microscope is being able to focus on a fixed point of living cells while offering rejection of the background focal plane *(213)*. Modern confocal microscopes were first developed by an engineer named Marvin Minsky, and were later used in almost every biological laboratory *(4)*. Fluorescent microscopes began the initial wave for imaging at different resolutions. The fluorescent instrument was developed in the late 1970s, followed by the development of fluorescent strains in the early 1980s, which played a role in intracellular parameters. Once this fluorescent feature became popular, imaging became highly known in fields of biochemistry and electrophysiology *(4)*. The majority of research published in cell biology in the 1980s relied on fluorescence microscopy; for instance, fluorescence microscopes were used by researchers Osborne and Weber in 1982 to visualize cytoskeleton protein in cells (*142)*. The confocal microscope in 1979 focused on a main spot in the specimen, producing an optical sectioning effect in which the outer regions are completely eliminated *(231)*. The main point in which confocal imaging system focuses on in a sample, special

equipment is needed to form a 2-dimensional image by scanning the lightened spot over the specimen *(4)*. The first research was published in 1985 by a team of researchers who obtained a series of nuclei images in which chromatin was stained with a fluorescent color *(4)*. The advantage confocal has over other conventional microscopes is the ability to eliminate background resolutions from collected images and focus on the main subject area while also providing high quality images *(168)*.

The confocal microscope has several advantages over traditional microscopes for studying the interaction between bacteria and food *(201)*. It has been used to study the colonization and attachment of microorganisms in a hydrated plant and animal tissue cultures *(68)*. Another advantage of the confocal imaging system is the ability to follow changes over time such as the food structure development or changes in bacterial populations during a process *(201)*. The capability to determine the biological state of microorganisms without disrupting their connection with the food matrix can be useful in determining the means by which microorganisms survive *(201)*. Another advantage involves the dryness of samples, for example, it is crucial for Scanning Electron Microscopy (SEM) that the sample be dry or else the image will come out poorly; however, with confocal it is not necessary *(74)*. Other advantages consist of being able to control the field depth and the ability to collect optical segments from thick samples *(60)*.

Relative to food safety, *Salmonella* and *E. coli* O157:H7 have been known to colonize and migrate within the edible portion of the plant, such as the leaf and stem, as well as the inedible portion such as the seeds and roots as determined by laser scanning confocal microscopy *(157)*. Prior to the spinach outbreak in 2006, researchers have studied

the distribution of *E. coli* O157:H7 among spinach plants to determine whether the organism would stay in the soil system or move through the stem to the leaves. The study indicated that *E. coli* was able to survive up to 28 days in the soil as well as migrate from the soil through the roots but was not able to travel past the root system *(21)*. It was concluded that bacterial contamination is less likely to move from the soil surface to the edible portions of the plant. Another study used confocal scanning laser microscopy to evaluate the site where *E. coli* O157:H7 was attached to the lettuce leaves *(194)*. *E. coli* O157:H7 was found attached to the exterior surfaces, trichomes, stomata, and cut edges of the leaves *(194)*. *Salmonella* plant-interaction was also observed on iceberg leaves in light and dark incubation using the confocal microscope *(131)*. In the light, *Salmonella* was observed near the stomata opening and in the inner leaf tissues. In contrast, the dark caused the scattered attachment and poor internalization of the stomata.

The confocal microscope is a unique tool that has several advantages over the traditional microscopes. It has become one of the most preferred technique in determining interactions between microorganisms and living tissue. Furthermore, in regard to food safety, the confocal microscope can be a useful tool in determining the relationship between pathogenic bacteria and the food matrix.

CHAPTER III

MATERIALS AND METHODS

General

Bacterial Cultures

Rifampicin-resistant (Rif+) isolates of *Salmonella* corresponding to serotypes Typhimurium, Saintpaul, and Agona, and Rif+ Shiga toxin-producing *E. coli* isolates corresponding to serotypes O104:H4, O111:H1, and O157:H7 were obtained from the Food Microbiology Culture Collection (Texas A&M University, College Station, TX). Each isolate was stored at -80 °C on cryocare vials (Key Scientific Products, Round Rock, TX). Prior to experimentation, each culture was revived by separating one bead from the cryocare bank and transferring to a test tube containing 10 ml of sterile Tryptic Soy Broth (TSB, Difco, Sparks, MD), followed by incubating at 35 ºC for 18-24 h. A loopful of each culture was transferred to another sterile 10 ml TSB tube and incubated at 35 ºC for 18-24 h. A loopful of the last incubated TSB culture of each strain was streaked onto tryptic soy agar (TSA, Difco, Sparks, MD) slants and incubated at 35 ºC for 18-24 h. After incubation, slants were sealed with parafilm and stored at 4-7 ºC. Fresh working slants were prepared every 1-2 months. For identification, each of these cultures was streaked on TSA plates and incubated overnight at 35 ºC. The identity of isolates was confirmed by biochemical methods using Triple Sugar Iron Agar (TSI, BBL™, Sparks, MD) Urea test (BBL™) and Lysine Iron Agar (LIA, BBL™). Rifampicin resistance was verified by streaking on TSA

supplemented with 100 µg/L of rifampicin (Sigma-Aldrich Inc., St. Louis, MO) and incubation overnight at 35°C.

For the experiment involving confocal microscopy, a strain of *S.* Poona transformed to encode for red fluorescence protein (RFP) was obtained from the Food Microbiology Culture Collection. Plasmid stability was verified by overnight subculturing to TSB followed by plating on non-selective TSA agar and viewing under a fluorescent light (365 nm) (UVP Chromato Vue Cabinet and UV handheld lamp, Upland, FL). All colonies examined expressing RFP indicated plasmid stability. *Salmonella* was confirmed by biochemical methods as described above using TSI agar (BBL™) and Xylose Lysine Deoxycholate agar (XLD Difco, Sparks, MD). Cultures were preserved on TSA slants at 5°C. This microorganism was stored and revived prior to conducting the experiments as described above.

Inoculum Preparation

Salmonella and STEC strains were transferred from TSA slants into six 10-ml sterile TSB tubes using a sterile loop. The inoculated TSB tubes were incubated at 35 ºC for 18-24 h. After incubation, each strain was centrifuged three times (for washing of the cells) at 3,500 rpm for 15 minutes (Jouan B4i, Thermo Electron Corp., Madison, WI). After each cycle, the supernatant was discarded and the pellet was resuspended using peptone water (PW 0.1%, Sigma-Aldrich, St. Louis, MO). All bacterial suspensions were combined in a sterile 500-ml flask (KIMAX®, Rockwood, TN) to form a bacterial cocktail. The bacterial cocktail contained both *Salmonella* and STEC strains. For very high level inoculation (10 logs), 4 ml of culture was transferred to cell culture bottle (BD)

with a TSA surface of 75 cm^2. The inoculum was spread over the TSA surface by aseptically adding sterile glass beads followed by rotating beads over the entire surface. Six culture bottles per isolate were inoculated and incubated at 35°C for 24 h to obtain a bacterial lawn. Growth from each culture bottle was harvested by adding 10 ml of peptone water to each bottle, swirling the glass beads and then transferring the culture with a 10-ml pipette to a 15-ml conical centrifuge tube (VWR®, Radnor, PA). The suspensions were washed by centrifugation at 3,500 rpm for 15 minutes. After centrifugation *Salmonella* and STEC serotypes were combined to form a bacterial cocktail.

The bacterial concentration in the cocktails was determined by preparing serial decimal dilutions and spread-plating onto selective Lactose Sulfite Phenol Red Rifampicin agar (LSPR) for simultaneous *Salmonella* and STEC enumeration. LSPR is a selective medium that was developed to simultaneously enumerate *Salmonella* and STEC pathogens that are rifampicin resistant, also allowing a differential enumeration on the same sample *(41)*.

Seed Selection

Alfalfa, broccoli, clover, and mustard seeds were purchased from a local store in Bryan, Texas (Brazos Natural Foods, College Station, TX) and from a commercial supplier in Rushville, Nebraska (Tiensvold Farms). Seeds were stored at refrigeration temperature 5°C until use. Prior to inoculation, the original moisture content of the seed was determined by separating 2 g of seeds followed by subjecting to a_w measurements using a water activity meter (Aqualab series 3, Pullman, WA).

Seed Inoculation

Seeds (100g) were removed from storage and placed in a sterile bag (ZIPLOC®). Inoculation was accomplished by adding 10 ml of the bacterial cocktail using a pipette and pipette pump (Syringa, Boise, ID). Followed by hand massaging for 3 to 5 minutes. Non-inoculated seed samples were sampled as a control.

Methods of Inoculations

For experiments involving objective 1 the inoculum concentration for *Salmonella* and *E. coli* cocktail was 8 logs CFU/ml. For experiments involving objective 2 the inoculum concentration for *Salmonella* and *E. coli* cocktails was 8 and 10 log CFU/ml for high and very high levels and 4 and 5 logs CFU/ml for very low and low levels, respectively. For the production practices study, only low-level concentration was used and the concentration for *Salmonella* and STEC cocktail was 5 log CFU/ml. For experiments conducted within objective 3, the inoculum concentration of *Salmonella* and STEC cocktail was 8 log CFU/ml. For the purposes of this study, inoculum at 10 log CFU/ml will be defined as very high (VH), 8 log CFU/ml as high (H), 5 log CFU/ml as low (L), and 4 log CFU/ml as very low (VL).

To verify starting concentrations, preliminary studies were used to determine the concentration level by colony plate counting. After inoculation, seeds were sampled to determine water activity and counts of *Salmonella* and STEC concentration. During the drying period seed samples were tested every few hours until reaching the original a_w. The seeds were placed on sterile aluminum foil on a sterile tray and left overnight to dry in a biosafety cabinet (Esco Labculture Reliant™, Hatboro, PA) at room temperature

(25°C) until reaching the original a_w. After drying of the inoculated seeds, the concentration of each pathogen was 5 log CFU/g, for studies containing objective 1 and 3. For the objective 2, the concentrations for each pathogen were 1 – 3 log CFU/g for very low and low levels, respectively, and 5 and 8 log CFU/g for high and very high levels, respectively. The concentrations were estimated using serial dilutions plated on LSPR for objectives 1 and 2, and colonies for each pathogen were counted separately. For objective 3, *Salmonella* concentrations were estimated using serial dilutions plated on XLD.

Greenhouse

Alfalfa, broccoli, clover, and mustard microgreens were grown in four 182-cm x 121-cm (I.D. FHXUPR, College Station, TX) greenhouses inside an authorized biosafety level 2 (BL2) laboratory at Texas A&M. Maximum and minimum temperatures, and maximum and minimum relative humidity were measured using a temperature and humidity controller thermometer (Extech®, Boston, MA) throughout the growth period. Temperature and humidity were monitored daily. Lighting phases were set at a 12-h photoperiod of day and night using a 20-watt white fluorescent light (Phillips 27332-6, Garland, TX).

Sprouting and Harvesting

Ten g of inoculated alfalfa seeds were placed in sterile glass jars (Ball Model-550797441, College Station, TX) and soaked in 50 ml distilled water for 5 h at 25°C. After 5 h, the jars were drained using a micro sieve filter (Biodesigns™, New York, NY), followed by placing in a covered container and placement in the dark for 3 d to sprout.

During sprouting, seeds were rinsed with distilled water every day, and then exposed to light on day 4 to enhance germination and sampled on day 5. Seeds were rinsed by pouring 50 ml of distilled water into the glass jar, followed by draining the water through a micro sieve filter into a 1-L bucket containing 10% bleach (sodium hypochlorite), water for biohazard disposal of the contaminated water.

Microgreens Growth and Harvesting

Soil was sterilized in a Steris® autoclave (Mentor, OH) for 1 h at a temperature of 135ºC prior to planting. The effectiveness of the sterilization procedure was verified by plating autoclaved soil samples onto TSA, APC, coliform/*E. coli*, and yeast and mold 3M™ Petrifilim (3M™, Saint Paul, MN). Non-autoclaved samples served as a control.

Twenty-five g of inoculated seeds were placed on top of the soil surface in a 5x10-cm plastic planter (IEC©, CN-GAR-081, Danville, IL) containing sterile professional germination soil mix (Sungro® Model 900 RSI, College Station, TX) purchased from Texas A&M Borlaug Research Center. Seeds on the soil surface were sprayed with a mist of distilled water (300 um) three times using a spray bottle and covered for 4 d to allow germination. On day 4, tray covers were removed and microgreens were exposed to a white fluorescent light (1000bulbs F-20T12CW, Garland, TX) to allow growth.

Growth and Behavior of *Salmonella* and STEC on Alfalfa Sprouts and Microgreens

Harvesting

The growth period for alfalfa sprouts was set to 1 week, because sprouting took around 2-3 d followed by vegetative growth which took around an additional 3 d. Alfalfa

sprouts were sampled by taking the entire plant content (stem, roots, and seed coat) using sterile gloves and placing inside a stomacher bag.

The growth period for alfalfa microgreens before harvesting was set to 2 weeks, because the germination took around 4-5 d followed by vegetative growth which took an additional 9 - 14 d (Figure 1). Week 2 alfalfa microgreens were harvested by wearing sterile gloves to grasp the plants followed by cutting microgreens 2.5 cm above soil surface, which contained the upper and lower hypocotyl and the plants' cotyledons. Prior to cutting, scissors were sterilized by dipping in 100% pure alcohol (Koptec, King of Prussia, PA) and flamed using a Bunsen burner.

Sample Collection. Five g of the seeds, harvested sprouts, and microgreens samples were weighed and placed in separate stomacher bags containing 45 ml of 0.1% PW (1:10 dilution). The samples were pummeled in a stomacher (A.J. Seward, London, UK) for 1 minute. Serial dilutions were prepared using 0.1% PW and plated on LSPR, followed by incubation for 24 h at 35°C.

Experimental Design. This experiment followed a complete randomized design with three replications (n=3) and three triplicates of each replication (n=9) for seed inoculation, sprouting, and microgreen growth. Same batches of seeds inoculated at the indicated level (5 log) were used for both sprouting and microgreen growth. For sprouts and microgreens, 3 samples were taken from each single production element (sprout jars and planting trays). Counts of *Salmonella* and STEC on microgreens and sprouts were compared at the time of harvest to determine whether any effect was observed between sprouts and microgreens.

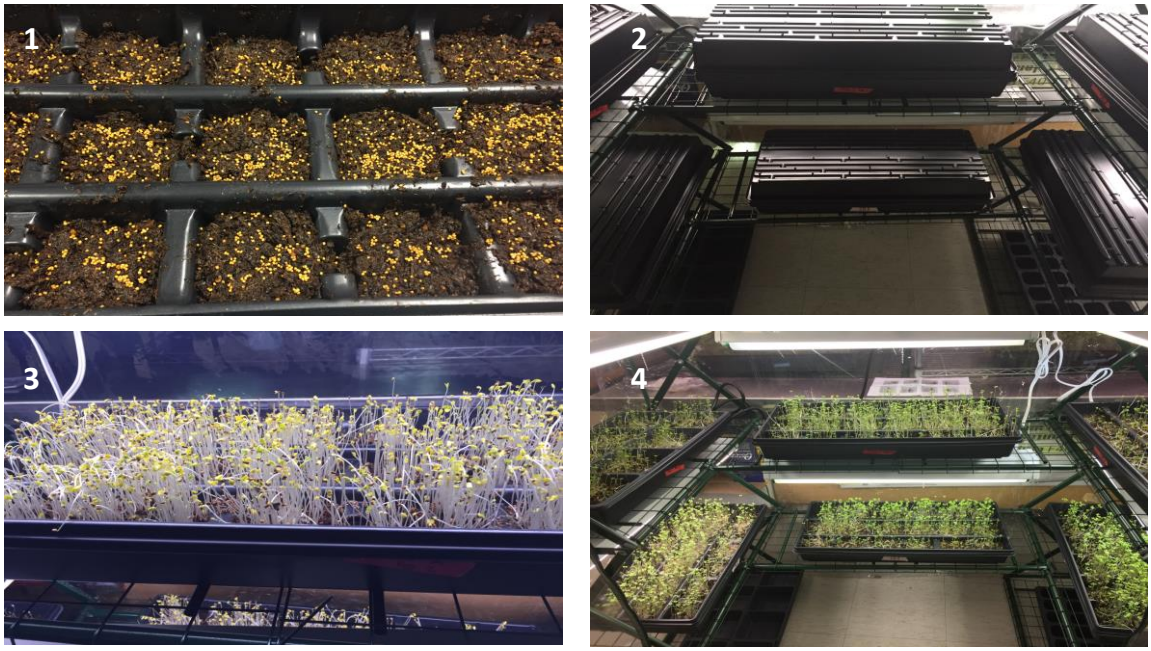

Figure 1. Production of Microgreens

Photos representing different stages of microgreens throughout the growth period

1: Planting of the seed

2: The Germination process (covering with a dark tray typically 4 d)

3: After germination (removal of trays and exposed to white florescent light) to allow for continued growth

4: Full grown microgreens

The Effect of Production Practices and Plant Type on the Ability of *Salmonella* and Shiga Toxin-Producing *E. coli* to Grow and Survive on Microgreens

Harvesting

Broccoli, clover, and mustard microgreens were grown as described above. Briefly, inoculated seeds were planted on sterile soil, covered to allow germination, and then exposed to white fluorescent light for continued growth. In these experiments, harvesting was conducted comparatively after 2 and 4 weeks of growth.

At each harvest time, the microgreens corresponding to each plant type were harvested using sterile gloves and scissors as described previously. Soil samples were also collected at 2 and 4 weeks from broccoli and clover microgreen trays to determine the role of soil as a possible source of contamination. Microgreens were harvested as described previously, cutting 2.5 cm above the soil surface, which includes the upper and lower hypocotyl and the leaves. The remaining plant parts (roots) and soil were collected for microbial enumeration.

For the production practice study, microgreens samples were harvested as previously described above, cutting 2.5 cm and also 6.5 cm above the soil surface. 2.5 cm contained both lower and upper hypocotyl (shoot) and leaves, while 6.5 cm only contained upper hypocotyl and leaves.

Sample Collection. Sample collection of microgreens and soil is similar to methods previously described. Five g of samples were placed in a stomacher bag containing 45 ml of 0.1% PW. Samples were then pummeled for 1 minute, followed by the preparation of serial dilutions using 0.1% PW, and then plated on LSPR for

enumeration of *Salmonella* and *E. coli* colonies. Plates were then incubated for 24 h at 35°C to allow for growth.

Experimental Design. The effect of harvest period was determined by enumerating populations of *Salmonella* and Shiga toxin-producing *Escherichia coli* on microgreens and in the soil at different weeks of growth. Another experiment was conducted to determine the dynamics of counts of *Salmonella* and STEC as a function of different harvest practices on microgreens. Counts were conducted by plating serial dilutions onto LSPR agar. Both experiments were conducted in three replications (n=3), with three triplicates of each replication (n=9). Harvest period followed a 3x2 factorial design and production practices followed a 2x2x2 factorial design. Three to five samples taken from one planting tray was considered as one replicate. The counts of *Salmonella* and STEC on the three plant types were also compared to determine if there was any difference among broccoli, clover, and mustard microgreens in regard to bacterial numbers.

Statistical Analysis

All bacterial counts from all studies were converted to log values before the statistical analysis. Statistical analysis was conducted using one-way ANOVA using JMP v10.0.0 (SAS Institute Inc., Cary, N.C.). Significant difference ($P<0.05$) among mean values was conducted using Tukey's studentized range test.

Evaluation of *S.* Poona Distribution on Microgreens Using a Confocal Microscope

Harvesting

Clover and mustard microgreens were grown by planting inoculated seeds on sterile soil, followed by covering to allow germination, then exposing to white fluorescent light for continued growth. The growth period before harvesting of microgreens was set to 2 and 4 weeks.

At 2 and 4 weeks, mustard and clover microgreens were harvested by methods described above. Each plant was dissected into two sections: the edible (above the cotyledons, and middle shoot) and inedible (below cotyledon and seed coats) portions. The edible portions were examined at 6.5 cm above soil surface for middle shoots, and 7.5 cm above soil surface for leaves. The inedible portions involved seed coats and lower shoots (below cotyledon, 2.5 cm above the soil surface). Microgreens were dissected using sterile tweezers, and individual plants were dissected by pulling the entire plant from the soil, followed by covering with aluminum foil and placing in a gallon-sized storage bag (ZIPLOC®, SC Johnson, Racine, WI). Each storage bag containing the dissected microgreens was placed in a closed cooler prior to transportation to Texas A&M University Imaging laboratory. The maximum time between harvesting and arrival was 15-30 min. After arrival at the imaging facility, plants were dissected into appropriate sections (edible and inedible portions), as shown in Figure 2, for viewing under the microscope.

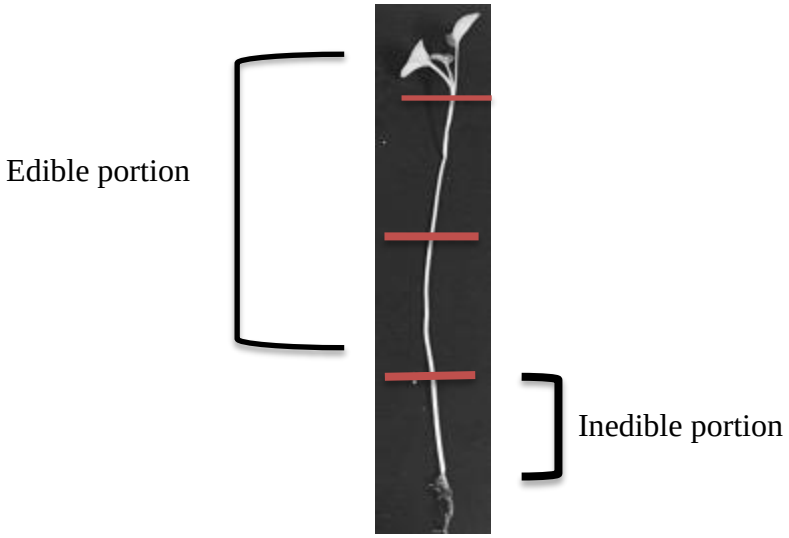

Figure 2. Dissection of plant parts for confocal microscope observation

Confocal Laser-Scanning Microscope

Samples were taken to Texas A&M University College of Veterinary Medicine Image Analysis Laboratory (College Station, TX) to examine *S.* Poona distribution using a confocal microscope. Each plant was dissected in distinct lengths (2.5, 6.5, and 7.5 cm) using a sterile surgical blade (Swann-morton®, England). Microgreen plants were directly placed on 25x60-1 mm (Fisherbrand®, Pittsburgh, PA) micro cover glass slides, followed by covering with plastic cover slips (fisher scientific, S175222, Waltham, MA), and then viewed under the microscope.

Zeiss 510 META confocal microscope uses two confocal channels, one spectral detection channel (META), two channels non-descanned detection, and one transmitted light channel. The software used was FRET and FRAP software.

Dimensions. A Hene laser was used with a 561-nm excitation. *S.* Poona was excited using 561 nm laser line. Emission was collected using 579-624 nm. The image dimension for the confocal was 212.47µm x 2.12.47µm. Images were collected using a 40x /1.4 NA oil objective and samples were collected from 1 µm section of thickness.

Experimental Design. Clover and mustard seeds were inoculated with 8 log CFU/ml *S.* Poona RFP cells as described previously, and examined at 2 and 4 weeks of growth with a confocal microscope to determine which portions (edible or inedible) of the microgreens were colonized with the target organism. Observation of microgreens using a confocal microscope was conducted in two replications (n=2), and at least three samples were observed each time. Samples collected from same tray was considered as one replicate. For quantitating the levels of *S.* Poona associated with edible and inedible

portions of microgreens using the confocal imaging system, three randomly selected fields for each sample were captured (images) and the number of *Salmonella* cells were enumerated using an image processing program (Imagej, Bethesda, Maryland). This experiment was conducted in two replicates (n=2). The size of the field was calculated using a stage micrometer (Fisher, Pittsburgh, PA) and the counts of *Salmonella* were expressed on cells per μm^2 basis.

CHAPTER IV

RESULTS AND DISCUSSION

Growth and Behavior of *Salmonella* and STEC on Alfalfa Sprouts and Microgreens

The growth of both *Salmonella* and STEC on microgreens and sprouts is shown in Table 2. Counts of *Salmonella* and STEC on the seed prior to planting were 5.6 log and 5.1 log CFU/g, respectively. *Salmonella* and STEC were not detected on non-inoculated seed samples. Pathogenic bacteria were found on the sprouts and on the microgreens at counts ranging from 6.8 to 8.5 log CFU/g at the time of harvesting.

Table 2 shows the counts of *Salmonella* and STEC on sprouts and microgreens. *Salmonella* on alfalfa sprouts increased 2.8 log CFU/g from the contaminated seed, confirming growth had occurred during sprouting as has been reported by the FDA *(83)*. Concentrations of *Salmonella* on sprouts reached 8.5 log CFU/g. In contrast, the increase in counts of *Salmonella* on microgreens was smaller than sprouts 7.6 log CFU/g ($P<0.05$). Therefore, results indicated growth of *Salmonella* occurred during sprouting and to a lesser extent during microgreens germination.

Like the *Salmonella*, STEC on alfalfa sprouts increased 2.6 log CFU/g, from the contaminated seed, also indicating growth occurred during sprouting ($P<0.05$). Counts of STEC reached 7.2 log CFU/g on alfalfa sprouts. Increase of STEC on microgreens was significantly less than on sprouts ($P<0.05$). Mean counts of STEC on microgreens reached 6.8 log CFU/g. As previously stated for *Salmonella*, even though there was growth of STEC on microgreens, it was significantly smaller than on sprouts ($P<0.05$).

Table 2. Mean counts (log CFU/g)[b] of *Salmonella* and STEC on alfalfa sprouts and microgreens

Target Organisms	Seed[a]	Sprouts	Microgreens
Salmonella	5.6	8.5^{A}	7.6^{B}
STEC	5.1	7.2^{A}	6.8^{B}

[a] Seed indicates the initial level of concentration of target organisms found on the seed prior to planting. Bacterial cocktail of *Salmonella* and STEC populations were inoculated on alfalfa seeds to a target of 5.6 log (*Salmonella)* and 5.1 (STEC) log CFU/g prior to planting. Sprouts were harvested on day 5, and microgreens on day 14.

[b] Values with different superscripts within rows differ statistically ($P<0.05$).

Sprouts and microgreens counts of *Salmonella* were significantly higher than STEC, 8.5 log CFU/g and 7.6 log CFU/g ($P<0.05$), respectively. Similar results were found on sprouts by Charkowski et al. *(55)*, who tested the difference of growth for *S.* Newport and *E. coli* O157:H7 on alfalfa sprouts. Their results also indicated that both pathogenic bacteria were able to grow on alfalfa sprouts; however, *S. enterica* serotypes contained higher counts than *E. coli* O157:H7. Their research was later expanded to determine the difference in attachment of *S. enterica* serotypes and *E. coli* O157:H7 to alfalfa sprouts *(15)*. The results revealed that *E. coli* O157:H7 (<10 CFU/g), using an attachment assay, was not able to attach as well as *S. enterica* serotypes and other plant-associated bacteria (*Pseudomonas*) to alfalfa sprouts. Barak et al *(15)* also reported that the removal of most *E. coli* O157:H7 cells from alfalfa sprouts occurred after water rinsing. One characteristic that they observed that may support this observation is the difference in fimbria (curli) among *S. enterica* and *E. coli* O157:H7. Curli fimbriae are extracellular fibers used for attachment, which are produced by many *Enterobacteriaceae* organisms, including enteric *Salmonella* and *E. coli (17).* In order for the curli expression to occur, csg operons are required *(14, 166)*; however, more than half (>95%) of single base pairs of *csgD* promoter modifications leave *E. coli* O157:H7 without the curli fimbriae *(211)*. Therefore, curli may be a reason *S. enteria* is able to attach better to sprouts *(182)*, and may also be the reason why majority of sprout-related outbreaks are linked to *S. enterica* and not enteric *E. coli (82)*. Although the observation seen in the study conducted by Barak et al *(15)* is similar to what is presented in this current research for

sprouts; however, for microgreens this may also be true, but further research would need to be conducted to determine this accuracy.

In general, both *Salmonella* and STEC inoculated onto alfalfa seeds grew during sprouting and to lesser extent during germination for microgreens. Multiple studies have reported aerobic plate counts (APCs) as high as 6 - 8 log CFU/g in sprouts due to the production process *(83, 171).* A similar trend was also observed by Xiao et al. *(235),* whose results indicated *E. coli* O157:H7 was able to grow to 3.2-5.1 logs on radish sprouts, and to a lesser extent on microgreens. The result of higher pathogenic growth on sprouts than microgreens could be primarily due to high humidity, high temperature, and constant watering during sprout production, as well as the distribution of pathogenic bacteria on other parts of the plant when watering. The warm temperature, high moisture content, and nutrient availability are all factors that are considered favorable for the growth and survival of pathogenic bacteria *(112, 215).* In fact, other researchers like Laborde et al. *(133)* have concluded that the abundant nutrients released, high moisture levels, and the heat generated during the sprouting process help ensure the growth and survival of pathogenic microorganisms.

The role of nutrient availability in the growth and survival of pathogenic bacteria in sprouts was explored by Hamilton and Vanderstoep *(104),* who demonstrated that alfalfa seeds and sprouts contained more carbohydrates and proteins than other vegetables such as lettuce and cabbage. Other researchers also reported that the concentration of various nutrients was 40 times higher in microgreens than mature plants *(225),* thereby making sprouts and microgreens an ideal substrate for bacterial growth.

In conclusion, this study indicated that sprouts were able to harbor higher levels of pathogenic bacteria compared to microgreens, possibly due to its production (high humidity, high temperature, and constant water availability) *(83, 86, 235)*. Microgreens also showed significant growth of target microorganisms, but to a lesser extent compared to sprouts. This increase in bacterial growth on microgreens could have occurred during the germination process where temperature and humidity levels were high. The high levels of contamination occurring during sprouting and, to a lower extent, germination, would imply the importance of minimizing contamination during these processes is crucial. Additionally, since neither process include a step for the elimination of bacterial pathogens, the prevention of seeds from becoming contaminated is therefore an essential step for ensuring the safety of sprouts and microgreens. The FSMA Final Rule on Produce Safety (Standards for the growing, harvesting, packing, and holding of produce for human consumption) (2017) specific for sprouts (subpart M), requires taking actions to prevent the introduction of bacterial pathogens onto seeds used for sprouting, such as either treating seeds using a scientific approach or relying on prior treatment provided by the grower, supplier, or distributor *(86)*. However, since microgreens are typically grown in soil/substrate, they are not subject to same requirements as sprouts (subpart M). Furthermore, they are still considered covered produce and unless exempt under the provisions in subpart A, all microgreen production farms are subject to all other subparts of the produce safety rule (CFR 80). Therefore, even though microgreens are not held to the same standards and may represent a lower risk than sprouts, producers should still implement GAPs to ensure the safety of microgreens for human consumption.

The Effect of Production Practices and Plant Type on the Ability of *Salmonella* and Shiga Toxin-Producing *E. coli* to Grow and Survive on Microgreens

The objective of this study was to determine if production practices such as the harvest date and the height of the plants at harvest has an effect on the growth and survival of *Salmonella* and STEC on broccoli, clover, and mustard microgreens. As previously described, soil was also sampled to pinpoint the likely source of contamination. Instead of testing all three plants, only broccoli and clover microgreens were selected for soil sampling, because between the two, one gave higher counts of both pathogenic bacteria while the other gave lower counts of both pathogens. Possibly, the amount of contaminated seed coats still attached to broccoli microgreens and not clover microgreens at the time of harvesting could be the reason differences in counts were observed between sample types.

Harvest Period

***Salmonella* Growth and Survival on Microgreens**. The growth and survival of *Salmonella* on mustard, broccoli, and clover microgreens at 2 and 4 weeks produced from contaminated seeds at varying initial inoculum levels is presented in Table 3. Counts of *Salmonella* on mustard seeds prior to planting were 8.2, 5.5, 3.2 and 2.1 log CFU/g for very high (VH), high (H), low (L), and very low (VL). At week 2 of microgreen growth, mean counts of *Salmonella* for VH, H, L, and VL on mustard microgreens were 7.4, 6.7, 6.4, and 5.2 log CFU/g, respectively. All inoculation levels expect for VL ($P>0.05$) had significantly lower concentrations of *Salmonella* after 4 weeks of microgreen growth (6.7, 5.6, 5.4, and 4.9 log CFU/g) compared to 2 weeks of growth ($P<0.05$). The log reduction

Table 3. Mean counts (log CFU/g)[a] of *Salmonella* on broccoli, mustard, and clover microgreens at two and four weeks

Microgreens				
	Broccoli			
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	8.7	7.8^{A}	6.4^{B}	0.164
High	4.3	6.9^{A}	6.4^{B}	0.128
Low	2.8	6.9^{A}	5.7^{B}	0.178
Very Low	1.0	6.2^{A}	5.2^{B}	0.128
Mustard				
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	8.2	7.4^{A}	6.7^{B}	0.136
High	5.5	6.7^{A}	5.6^{B}	0.132
Low	3.2	6.4^{A}	5.4^{B}	0.146
Very Low	2.1	5.2^{A}	4.9^{A}	0.142
Clover				
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	8.1	7.6^{A}	5.6^{B}	0.125
High	6.0	7.4^{A}	6.4^{B}	0.124
Low	2.4	6.7^{A}	4.3^{B}	0.116
Very Low	1.8	6.1^{A}	3.8^{B}	0.156

[a] Means within each row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM indicates the standard error mean (n=3).

[b] Seed represents the initial concentration of *Salmonella* for each concentration on the seed prior to planting.

of *Salmonella* at 4 weeks was 0.3-1.0 for low inoculation levels, and 0.7-1.1 for high inoculation levels.

Counts of *Salmonella* on broccoli seeds (Table 3) prior to planting were 8.7, 4.3, 2.8, and 1.0 log CFU/g, respectively, for VH, H, L, and VL. Mean counts of *Salmonella* on broccoli microgreens at 2 weeks of growth, for VH, H, L, and VL inoculum levels were 7.8, 6.9, 6.9, and 6.2 log CFU/g, respectively. Broccoli microgreens had significantly lower concentrations of *Salmonella* at 4 weeks when compared to 2 weeks for all concentration levels with counts of 6.4, 6.4, 5.7, and 5.2 log CFU/g, respectively ($P<0.05$). During week 4 of growth, there was a reduction of 1.0-1.2 log for low levels and 0.5-1.4 log for high levels. As seen with mustard microgreens, the concentration of *Salmonella* decreased significantly after 2 weeks. This reduction could be due to the fewer seed coats still attached to microgreens after the 2 weeks of growth.

Counts of *Salmonella* on clover seeds prior to planting were 8.0, 6.1, 2.4, and 1.8 log CFU/g, respectively for VH, H, L, VL (Table 3). Mean counts on clover microgreens at 2 weeks for VH, H, L, and VL inoculum levels were 7.6, 7.4, 6.1, and 6.7 log CFU/g, respectively. At 4 weeks clover microgreens had significantly fewer *Salmonella* for both high and low levels when compared to 2 weeks with 6.4, 5.6, 4.3, and 3.8 log CFU/g ($P<0.05$). This indicated that after 2 weeks, *Salmonella* was reduced by 2.3-2.4 logs for low levels, and 1.0-2.0 logs for high levels. As previously mentioned, this reduction could be due to the fewer amount of seed coats attached to the leaves at 4 weeks than compared to 2 weeks.

Continued growth of clover baby greens at 6 weeks was also analyzed to examine whether the bacterial numbers would continue to decline. Clover was the only commodity chosen for further examination because it gave greater reduction in both target organisms than compared to both broccoli and mustard microgreens. The reduction of *Salmonella* at 6 weeks are presented in Table 4. Counts for *Salmonella* for VH, H, L, and VL were 4.6, 4.5, 2.9, and 1.6 log CFU/g, respectively. Regardless of initial contamination level, *Salmonella* counts were significantly lower at 6 weeks compared 2 and 4 weeks ($P<0.05$). This indicated a 3-log reduction for high levels and a 3.8-4.5-log reduction for low levels at 6 weeks, thereby suggesting time plays a role in the bacterial reduction seen in plants.

Soil. The growth and survival of *Salmonella* in the soil sampled from broccoli and clover trays at 2 and 4 weeks is presented in Table 5. There was no detection of any microorganisms in the soil prior to planting. At 2 weeks, mean counts of soil samples for VH, H, L, and VL were 8.3, 6.3, 7.0, and 6.3 log CFU/g, respectively. When compared to the broccoli microgreen bacterial counts, there were no significant differences observed between the associated soil and microgreens samples for H, L, and VL inoculum levels ($P>0.05$). At 4 weeks, mean counts were 7.9, 6.4, 5.8, and 6.2 log CFU/g for VH, H, L, and VL, respectively. There were also no significant difference of *Salmonella* counts observed between broccoli soil and microgreens for majority of inoculum levels at this time ($P>0.05$). A possible explanation for the no observed difference at 2 and 4 weeks is that the majority of broccoli microgreens had seed coats still attached at harvest time. Those seed coats that did drop to the soil surface during the growing period may have contaminated the soil and could explain the lack of observed difference in counts between

Table 4. Mean counts (log CFU/g)[a] of *Salmonella* found on microgreens and baby greens

Clover					
Inoculum level		**Week 2**	**Week 4**	**Week 6**	**SEM**
	Seed[b]	**Microgreens**	**Microgreens**	**Baby Greens**	
Very High	8.1	7.6^{A}	5.6^{B}	4.6^{C}	0.109
High	6.0	7.4^{A}	6.4^{B}	4.5^{C}	0.147
Low	2.4	6.7^{A}	4.3^{B}	2.9^{C}	0.165
Very Low	1.8	6.1^{A}	3.8^{B}	1.6^{C}	0.129

[a] Means within each row, at each time, and with different superscripts are significantly different. SEM represents the standard error mean (n=3).

[b] Seed represents the initial concentration of *Salmonella* for each concentration level on the seed prior to planting. Weeks 2 and 4 represent microgreens and week 6 represents baby greens.

Table 5. Mean counts (log CFU/g)[a] of *Salmonella* in soil and broccoli and clover microgreens at two and four weeks

Microgreens						
Broccoli						
Inoculum level		**2 Weeks**		**4 Weeks**		
	Seed[b]	**Microgreens**	**Soil**	**Microgreens**	**Soil**	**SEM**
Very High	8.7	7.8^{B}	8.3^{A}	6.4^{A}	7.9^{B}	0.125
High	4.3	6.9^{A}	6.3^{A}	6.4^{A}	6.4^{A}	0.132
Low	2.8	6.9^{A}	7.0^{A}	5.7^{A}	5.8^{A}	0.133
Very Low	1.0	6.2^{A}	6.3^{A}	5.2^{A}	6.2^{B}	0.233
Clover						
Inoculum level		**2 Weeks**		**4 Weeks**		
	Seed[b]	**Microgreens**	**Soil**	**Microgreens**	**Soil**	**SEM**
Very High	8.1	7.6^{A}	8.1^{A}	5.6^{A}	7.8^{B}	0.135
High	6.0	7.4^{B}	8.0^{A}	6.4^{A}	7.6^{B}	0.125
Low	2.4	6.7^{A}	7.2^{A}	4.3^{A}	6.6^{B}	0.141
Very Low	1.8	6.1^{B}	7.2^{A}	3.8^{A}	8.1^{B}	0.316

[a] Means within each row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM represents the standard error mean (n=3).

[b] Seed represents the target concentration of *Salmonella* for each concentration on the seed prior to planting.

plant and soil samples.

For clover at 2 weeks of growth for VH, H, L, and VL, mean counts were 8.1, 8.0, 7.2, and 7.2 log CFU/g, respectively (Table 5). When compared to clover microgreen counts, there was no difference between microgreens and soil for majority of inoculum levels ($P>0.05$). A possible reason as to why there was no observed difference in counts between microgreens and soil samples is, because most of clover microgreens had their seed coats still attached during this time. Those seeds coats that happen to drop to the soil surface during cultivation may have contaminated the soil which may explain the lack of observed difference in counts between plant and soil samples. At 4 weeks, there were higher counts of *Salmonella* in soil samples at 7.8, 7.6, 6.6, and 8.1 log CFU/g, respectively, than on microgreens at 5.6, 6.4, 4.3, and 3.8 log CFU/g, respectively, for all inoculum levels ($P<0.05$). There was a 1.2 and 2.2-log difference observed between soil and microgreen samples for high inoculum levels, and a 2.3 and 4.3-log difference between soil and microgreen samples for low inoculum levels. A possible explanation is that after 2 weeks of growth, the seed coats begins to drop from the leaves causing contamination of the soil. After being introduced to the soil, the soil can become a pathogens reservoir *(89),* because it is providing nutrients, moisture, and warm temperature for continued bacterial growth. However, the counts on the microgreens are reduced during week 4 of growth, because the seed coats begin to detach earlier on in the growth period (around 2 weeks). This could explain the lower counts of *Salmonella* observed on the plant than compared to the soil at 4 weeks, because an ample amount of time has passed since the seeds dropped from the leaves causing little contamination on

the plant. The soil however, is providing a favorable condition for the continued growth and survival of the microorganisms. Semenov et al. *(192)* described the survival and growth of enteric pathogens is greatly influenced by the soil components for instance, the availability of substrates, pH, moisture content, and temperature are all factors that are considered favorable for the growth and survival of pathogenic bacteria.

These findings for both broccoli and clover are important since they indicate a possible primary source of contamination for microgreens. Broccoli microgreens still have most seed coats attached during 4 weeks of growth, while attached seed coats on clover microgreens were rarely observed. The difference observed in *Salmonella* counts between clover microgreens and soil associated samples, contrasted with dissimilar counts for broccoli microgreens and associated soil, may indicate that seed coats are a primary source of contamination of microgreens.

STEC Growth and Survival on Microgreens. The growth and survival of STEC on mustard, broccoli, and clover microgreens at 2 and 4 weeks is presented in Table 6. Counts of STEC on mustard seeds prior to planting were 8.2, 5.1, 2.7 and 1.0 log CFU/g, respectively for VH, H, L, and VL. Average counts of STEC at 2 weeks for VH, H, L, and VL inoculum levels were 6.8, 6.2, 5.7, and 4.2 log CFU/g, respectively. At 4 weeks, counts of STEC were significantly lower than compared to 2 weeks for both high and low inoculum levels 6.1, 5.1, 4.7, and 4.0 log CFU/g, respectively ($P<0.05$). A possible explanation could be fewer seed coats were observed still attached to microgreens at 4 weeks than compared to 2 weeks.

Table 6. Mean counts (log CFU/g)[a] of STEC on broccoli, mustard, and clover microgreens at two and four weeks

Microgreens				
Broccoli				
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	7.6	6.7^{A}	5.3^{B}	0.156
High	3.7	5.9^{A}	5.0^{B}	0.380
Low	2.7	5.6^{A}	4.3^{B}	0.120
Very Low	1.0	4.9^{A}	4.0^{B}	0.279
Mustard				
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	8.2	6.8^{A}	6.1^{B}	0.161
High	5.1	6.2^{A}	5.1^{B}	0.146
Low	2.7	5.7^{A}	4.7^{B}	0.146
Very Low	1.7	4.2^{A}	4.0^{A}	0.130
Clover				
Inoculum level	**Seed[b]**	**2 Weeks**	**4 Weeks**	**SEM**
Very High	7.5	6.7^{A}	4.7^{B}	0.179
High	5.2	6.6^{A}	5.3^{B}	0.157
Low	1.7	5.5^{A}	3.6^{B}	0.073
Very Low	1.8	4.9^{A}	2.8^{B}	0.135

[a] Means within each row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM represents the standard error mean (n=3).

[b] Seed represents the initial concentration of STEC for each concentration level on the seed prior to planting.

Counts of STEC on broccoli seeds prior to planting were 7.6, 3.7, 2.7 and 1.0 log CFU/g, respectively for VH, H, L, and VL (Table 6). Broccoli microgreens at 2 weeks contained STEC counts for VH, H, L, and VL at 6.7, 5.9, 5.6, and 4.9 log CFU/g, respectively. STEC decreased significantly at 4 weeks, and counts were 5.3, 5.0, 4.3, and 4.0 log CFU/g for VH, H, L, and VL, respectively ($P<0.05$), a 0.9 to 1.4-log decrease for high inoculum levels and a 0.9 to 1.3-log decrease for low levels.

Counts of STEC on clover seeds prior to planting were 7.5, 5.2, 1.7 and 1.8 log CFU/g, respectively for VH, H, L, and VL (Table 6). At week 2, mean counts of STEC on clover microgreens for VH, H, L, and VL were 6.7, 6.6, 5.5, and 4.9 log CFU/g, respectively. There was a significant decrease during week 4 for both high and low levels (4.7, 5.3, 3.6, and 2.8 log CFU/g) levels, respectively. This data indicated a 1.3-2.0-log decrease of STEC for high levels and a 1.9-2.1-log decrease for low levels.

The continued growth of clover baby greens at 6 weeks was also observed. The reduction of STEC at 6 weeks is presented in Table 7. Counts for STEC for VH, H, L, and VL were 3.9, 3.6, 1.9, and 1.0 log CFU/g, respectively. H, L, and VL counts were significantly lower at 6 weeks compared to 2 and 4 weeks ($P<0.05$). There was significant difference observed between week 2 and 4 for VH; however, there was no significant difference observed between week 4 and 6 ($P>0.05$). A 2.8-3.0-log reduction was noted for high levels and a 3.6-3.9-log reduction for low levels, indicating that the time of growth plays a role on the bacterial reduction seen in plants.

Soil. The growth and survival of STEC in the soil sampled from broccoli and clover trays at 2 and 4 weeks is presented in Table 8. There was no detection of any

Table 7. Mean counts (log CFU/g)[a] of STEC found on microgreens and baby greens

Clover					
Inoculum level		**Week 2**	**Week 4**	**Week 6**	**SEM**
	Seed[b]	**Microgreens**	**Microgreens**	**Baby Greens**	
Very High	7.5	6.7^{A}	4.7^{B}	3.9^{B}	0.165
High	5.2	6.6^{A}	5.3^{B}	3.6^{C}	0.160
Low	1.7	5.5^{A}	3.6^{B}	1.9^{C}	0.073
Very Low	1.8	4.9^{A}	2.8^{B}	ND	0.101

[a] Means within each row, at each time, and with different superscripts are significantly different. SEM represents the standard error mean (n=3).

[b] Seed represents the initial concentration of STEC for each concentration level on the seed prior to planting. Weeks 2 and 4 represent microgreens and week 6 represents baby greens.

Table 8. Mean counts (log CFU/g)[a] of STEC in soil and broccoli and clover microgreens at two and four weeks

Microgreens						
Broccoli						
Inoculum level		**2 Weeks**		**4 Weeks**		
	Seed[b]	**Microgreens**	**Soil**	**Microgreens**	**Soil**	**SEM**
Very High	7.6	6.7^{B}	7.7^{A}	5.3^{B}	7.2^{A}	0.144
High	3.7	5.9^{A}	6.3^{A}	5.0^{A}	5.4^{A}	0.125
Low	2.7	5.6^{B}	6.4^{A}	4.3^{A}	4.6^{A}	0.117
Very Low	1.0	4.9^{B}	5.7^{A}	4.0^{B}	5.1^{A}	0.191
Clover						
Inoculum level		**2 Weeks**		**4 Weeks**		
	Seed[b]	**Microgreens**	**Soil**	**Microgreens**	**Soil**	**SEM**
Very High	7.5	6.7^{A}	7.3^{A}	4.7^{A}	7.1^{B}	0.164
High	5.2	6.6^{A}	7.2^{B}	5.3^{A}	6.7^{B}	0.137
Low	1.7	5.5^{A}	5.9^{A}	3.6^{A}	5.6^{B}	0.132
Very Low	1.8	4.9^{A}	6.3^{B}	2.8^{A}	7.1^{B}	0.312

[a] Means within each row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM represents the standard error mean (n=3).

[b] Seed represent the target concentration of STEC for each concentration level on the seed prior to planting.

microorganisms in the soil prior to planting. Counts at week 2 for VH, H, L, and VL were 7.7, 6.3, 6.4, and 5.7 log CFU/g, respectively. There were higher counts of STEC in soil VH, L, and VL at 7.7, 6.4, and 5.7 log CFU/g, respectively than on microgreens at 6.7, 5.6,and 4.9 log CFU/g, respectively ($P<0.05$). There was a 1-log difference between soil and microgreens for VH, and a 0.8-log difference between soil and microgreens for L and VL inoculum levels. A possible explanation for this may be once seed coats detach from leaves onto soil surface, the microorganisms present on seed coats may transfer to soil. As previously mentioned, soil can be a reservoir for pathogenic growth *(89)* due to favorable conditions such as, nutrients, temperature, moisture content, and pH. Therefore, this may explain the high STEC counts observed in soil than microgreens samples. The survival, replication, and movement of *E. coli* O157:H7 within the soil has been studied extensively *(88, 89, 192)*. For example, Gagliardi et al. *(89)* evaluated the persistence of *E. coli* O157:H7 in the soil and on plant roots. Results indicated the presence of *E. coli* O157:H7 in soil samples persisted up to 41 d and suggested it could be due to a number of factors such as, substrates and water found in the soil. Another reason for the high counts in the soil could be the temperature of the soil. Semenov et al. *(193)* study the influence of temperature on *E. coli* O157:H7 in soil amendments. The results indicated the greatest survival of *E. coli* O157:H7 occurred under low temperatures (7 and 16°C) than compared to high temperatures (23 and 33°C). Although the temperature of soil was not monitored during this current study, this could also be a reason for the high counts of STEC observed in the soil than compared to the microgreens. At 4 weeks, mean counts were 7.2, 5.4, 4.6 and 4.7 log CFU/g for VH, H, L, and VL, respectively. There were no significant

differences observed between soil and microgreens at this time point (P>0.05). Similar to *Salmonella,* this could be because broccoli microgreens still contain most of their seed coats at harvest. Those seed coats that dropped to the soil surface throughout the growth period contaminating the soil may be the reason why there was no observed difference in STEC counts between the plant and soil samples.

For clover, at week 2, VH, H, L, and VL mean counts were 7.3, 7.2, 5.9, and 6.3 log CFU/g, respectively (Table 8). There was a higher count of STEC observed for H and VL in soil samples at 7.2 and 6.3 log CFU/g, respectively, than in microgreens samples at 6.6 and 4.9 log CFU/g, respectively, at this time point (P<0.05). There was a 0.6 and 1.4-log difference observed between soil and microgreens for H and VL inoculum levels. As previously mentioned, a possible reason is that after the seeds drop from the plant onto the soil surface causing contamination. The pathogens are able to utilize nutrients and water in the soil causing them to proliferate, and therefore could explain the reason for observing higher counts in the soil than compared to the plant. However; there was no difference observed between microgreen and soil for VH and L inoculum levels. This could be because at 2 weeks, like broccoli, majority of clover microgreens still contain most of their seed coats at harvest. Those seed coats that dropped to the soil surface throughout the growth period contaminating the soil may be the reason why there was no observed difference in counts between the plant and soil samples. At 4 weeks, there were higher counts of STEC in soil samples at 7.1, 6.7, 5.6, and 7.1 log CFU/g, respectively, than on microgreen at 4.7, 5.3, 3.6, and 2.8 log CFU/g, respectively, for all inoculum levels (P<0.05). This difference as stated previously could be because, after week 2 of cultivation

contaminated seeds detach from leaves onto soil surface, causing microorganisms to transfer to the soil where resources are available for continued growth. However, during week 4 of growth counts from microgreens samples are reduced possibly, because minimal amount of seed coats are still attached to the plant. This could explain the difference in counts observed between the two sample types.

As previously mentioned, these findings for both broccoli and clover are significant, since they indicate a possible primary source of contamination for microgreens. Broccoli microgreens still have most of their attached seed coats at 4 weeks of growth, while attached seed coats on clover microgreens were rarely observed. The difference observed in STEC counts between clover microgreens and associated soil samples, contrasted with dissimilar counts for broccoli microgreens and associated soil may indicate that seed coats are a primary source of contamination for microgreens.

In general, growth of *Salmonella* on previously contaminated seeds occurred during the 2-week harvest period for broccoli, clover, and mustard microgreens for H, L, and VL inoculum levels. This increase could be likely due to the high humidity and warm temperature provided during the germination process. As stated in the first objective, high humidity and high temperature are factors that are favorable for bacterial growth *(112, 215)*. In this study, after seeds are sprayed with water for hydration and then covered with a dark dome (also sprayed with water), this causes an increase in temperature and humidity. While this process speeds up germination, it also creates a favorable environment for bacteria. Additionally, not only is the environment favorable, the access to nutrients from the leaves could be another reason for proliferation of the bacteria. For

example, the phyllosphere contains an abundant of nutrients that the pathogens can gain access, to continue to survive or sometimes grow *(146, 219)*. In addition, another justification as to why there was an increase could also be due to the high amount of seed coats still attached to all microgreens at 2 weeks. An explanation for the reduction of *Salmonella* at 2 weeks for VH inoculum level could be because the organism has already reached the maximum amount of bacteria that the population can sustain due to limited nutrients and; therefore, is approaching the death phase of the bacterial growth curve. A similar theory was proposed by Xiao et al. *(234)*, who also tested high and low levels of inoculums of *E. coli* O157:H7 on radish microgreens. Their study stated the reduction of *E. coli* O157:H7 from high level inoculums could be because the organism has already approached its growth limit, which they believed could result from nutrient exhaustion or by competitive microbiota present on the seed. Although, there have been theories as to why this reduction could occur, there is no current data supporting the reason for the reduction of high inoculum levels seen on plants during growth.

During week 4, *Salmonella* levels were significantly lower for broccoli, mustard, and clover microgreens compared to 2 weeks ($P<0.05$). Jablasone et al. *(116)* observed a similar trend of *S.* Typhimurium on lettuce, radish, and spinach over time (9 and 49 d), with significantly higher counts documented at 9 versus 49 d ($P<0.05$). This reduction could be because fewer seed coats were still present on the microgreens at 4 weeks compared to 2 weeks, especially for clover microgreens. Clover microgreens at 4 weeks had lower *Salmonella* concentrations compared to both broccoli and mustard microgreens. This observation could indicate that plant type may also play a role in the survival of

pathogenic bacteria, because broccoli and mustard shared similar bacterial counts, while clover had significantly less. This could be because after the 4th week harvest period, clover microgreens had fewer seed coats attached to their leaf, while broccoli and mustard seed coats were still attached (Figure 3). Furthermore, at 6 weeks, even though clover is considered a baby green, a significant reduction in *Salmonella* levels was observed compared to weeks 2 and 4 ($P<0.05$). This continued reduction could be related to the fact that rarely any seed coats were observed at the time of harvest and, because the environment was becoming unfavorable due to nutrient limitation, inconsistent temperature and humidity, or direct exposure to UV lights. Several studies have demonstrated the hostile environment like the ones listed above surrounding plants especially the leaves, and its effects on bacterial survival *(20, 137, 151, 219).* Mildred (*151)* studied the effect of exposure to UV radiation on the growth of plant and microorganisms. Results indicated that UV light damaged the outer membrane and DNA of bacteria, which resulted in cell death. Koper et al. *(130)* also studied the effect of UV light on *Pseudomonas syringae* found on plants. Results revealed cells experienced rapid death when in log phase than compared to stationary. The reason for this could be because when in stationary phase the repair mechanisms for protection against UV light are limited. Mercier and Lindow *(149)* examined the relationship between the abundance of carbohydrates on leaves on greenhouse grown bean plants and population sizes of *P. fluorescens.* Their results indicated a decrease in size over time after utilization of carbohydrates from leaves. Although this may apply to this current research; however, more data would need to be collected on the relationship between enteric pathogens and

1)

2)

Figure 3. Observation of seed coats present on broccoli, mustard, and clover microgreens at four weeks

Broccoli (1) and Clover (2) microgreens at 4 weeks. Mustard seed coats were similar to broccoli; however, its seeds coats were transparent making it difficult to view.

plant interactions during growth to determine this accuracy.

In a similar trend to that seen with *Salmonella,* STEC counts increased during week 2 for H, L, and VL inoculum levels for all microgreen types. Similar results were reported by Xiao et al. at the University of Maryland, who observed proliferation of both *E. coli* O157:H7 and O104:H4 after high and low-level inoculum on radish microgreens *(234)*. Just like *Salmonella,* STEC counts for VH inoculum level decreased during week 2 for all commodities (broccoli, clover, and mustard), indicating the bacteria may have already reached the maximum amount of bacteria that the population can sustain due to limited nutrients or toxic waste products being produced and; therefore, is approaching the death phase of bacterial growth. At week 4, STEC counts were significantly lower for all three microgreens ($P<0.05$). As discussed previously, this reduction in counts at week 4 could be due to fewer attached seed coats observed on the microgreens after 4 weeks compared to 2 weeks, and may also imply that seed coats could be the main point of contamination.

In addition to determining if the seed coats could be the main source of contamination, soil was tested from broccoli and clover trays. Soil collected from broccoli trays appeared to have similar *Salmonella* and STEC counts as the broccoli microgreens for majority of inoculum levels at 2 weeks, and for all inoculum levels at 4 weeks. As previously mentioned, the possible explanation for this could be because seed coats were still attached to broccoli microgreens during harvesting. Those seed coats that did drop to the soil surface throughout the growth period contaminating the soil may be the reason why there was no observed difference in counts between the plant and soil samples. Similar observation by Xiao et al. *(234)* also examined soil and radish microgreens

contaminated with *E. coli* O157:H7. Their data showed no significant difference was observed between the soil and edible portions of microgreens for high inoculum levels; however, no explanation was given as to why this difference occurred. As for clover, both *Salmonella* and STEC counts were higher in soil samples than in the microgreen samples at 4 weeks. This could be because at 4 weeks majority of seed coats were found on the soil surface and not on the plant which, could explain the difference in counts observed between the two sample types. Although the presence of bacterial pathogens in soil from vegetable crops has been extensively studied *(114, 115, 158),* the connection between soil and plant has not, and further research regarding the relationships of bacterial pathogens observed between plant and soil is needed.

Overall, from this study it was concluded both *Salmonella* and STEC grew on all microgreens after 2 weeks of cultivation for H, L, and VL inoculum levels, suggesting germination and seed coat play may be involved in this increase. This observation was also expressed by Xiao et al. *(234),* who reported that seed coats may play a role in the survival of *E. coli* O157:H7 on radish microgreens. After 4 weeks, a significant reduction was observed for both *Salmonella* and STEC populations on all microgreens ($P<0.05$). As stated previously, this reduction could be because of fewer attached seed coats on the microgreens at 4 weeks compared to 2 weeks. In addition, significant reductions were observed on clover baby greens at week 6 ($P<0.05$), suggesting time of cultivation supports bacterial reduction. Therefore, from observations in this study, it is likely that time of harvest plays a critical role in the growth and survival of enteric pathogens on

broccoli, clover, and mustard microgreens, and an extended harvest period may be beneficial in the reducing levels of pathogens.

Production Practices

***Salmonella* and STEC Growth and Survival on Broccoli Microgreens.** The growth and survival of *Salmonella* and STEC on broccoli microgreens at different harvest times is presented in Table 9. During week 2 of growth, counts of *Salmonella* from the 2.5-cm broccoli samples were significantly higher (4.7 log CFU/g) compared to 6.5-cm samples (4.4 log CFU/g). However, during week 4, there was a greater difference between the two harvest heights, namely 4.1 log CFU/g at 2.5 cm and 2.2 log CFU/g at 6.5 cm ($P<0.05$), a 0.3-log difference between the two harvest lengths at 2 weeks, and a 1.9-log difference between the two lengths at 4 weeks. The difference in counts observed between the two lengths could be related to when the seed coats falls from the leaf. If the seed coat becomes trapped within the lower stem, it can be collected with the sample. This is more likely to occur if seeds are spread densely prior to planting.

The STEC growth and survival on broccoli microgreens at different harvest lengths are also presented in Table 9. STEC counts on broccoli microgreens at week 2, like *Salmonella*, were significantly different when harvested at 2.5 cm (4.2 log CFU/g) compared to 6.5 cm (3.9 log CFU/g). In addition, at 4 weeks of growth, 2.5 cm also contained significantly higher counts of STEC (3.0 log CFU/g) compared to 6.5 cm (1.4 log CFU/g) ($P >0.05$). Similar to *Salmonella*, the difference in STEC counts observed between the two harvest lengths may be due to contaminated seed coats having more contact to the lower portions of the microgreens when compared to the upper portions. If

Table 9. Mean counts (log CFU/g)[a] of *Salmonella* and STEC on broccoli microgreens at different harvest lengths at two and four weeks

Target Organisms	**Broccoli Microgreens**					
		2 Weeks		**4 Weeks**		**SEM**
	Seed[b]	**2.5 cm**	**6.5 cm**	**2.5 cm**	**6.5 cm**	
STEC	2.3	4.2^{A}	3.9^{B}	3.0^{C}	1.4^{D}	0.061
Salmonella	3.0	4.7^{A}	4.4^{B}	4.1^{C}	2.2^{D}	0.104

[a] Mean within a row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM represents the standard error mean (n=3).

[b] Seed represents the initial count of target organisms on the seed prior to planting.

seeds are spread densely prior to planting the seed coat after falling from leaf, can become trapped within lower portions and; therefore, collected within the sample.

***Salmonella* and STEC Growth and Survival on Clover Microgreens**. The growth and survival of *Salmonella* and STEC on clover microgreens at different harvest lengths is presented in Table 10. At week 2, mean counts of *Salmonella* on clover microgreens at 6.5 cm were significantly lower (4.0 log CFU/g) than 2.5 cm (5.2 log CFU/g) ($P<0.05$). At week 4, there was also a significant difference (1.6 log) among the two harvest lengths with 3.7 and 2.1 log CFU/g for 2.5 cm and 6.5 cm ($P<0.05$), respectively. The 2.5 cm samples contained higher counts than the 6.5 cm sample, possibly because the 2.5 cm is closer to the soil surface where contamination is higher.

Counts of STEC for week 2 of growth on clover microgreens at 6.5 cm were significantly lower (3.5 log CFU/g) than at 2.5 cm (4.5 log CFU/g) by 1 log. During week 4 of growth, there was a 2.7-log reduction observed between 2.5 and 6.5 cm ($P<0.05$). The low counts of STEC on clover microgreens at 6.5 cm compared to 2.5 cm, as stated previously, could be due to more contaminated seed coats coming in contact with the lower stem of the microgreens compared to upper stem.

Overall, both *Salmonella* and STEC counts on broccoli and clover microgreens were significantly lower when microgreens were harvested more than 2.5 cm above the soil surface ($P<0.05$). Although there is limited data that evaluate bacterial numbers found on microgreens harvested 2.5 cm above soil surface *(234, 235)*, there does not appear to be any current data comparing different harvest lengths on bacterial survival. Therefore, this data provides important information that could be beneficial for the production of

Table 10. Mean counts (log CFU/g)[a] of *Salmonella* and STEC on clover microgreens at different harvest lengths at two and four weeks

Target Organisms	**Clover Microgreens**					
		2 Weeks		**4 Weeks**		**SEM**
	Seed[b]	**2.5 cm**	**6.5 cm**	**2.5 cm**	**6.5 cm**	
STEC	2.4	4.5^{A}	3.5^{B}	3.1^{C}	1.4^{D}	0.139
Salmonella	*3.4*	5.2^{A}	4.0^{B}	3.7^{C}	2.1^{D}	0.143

[a] Mean within a row, at each time, and with different superscripts are significantly different ($P<0.05$). SEM represents the standard error mean (n=3).

[b] Seed represents the initial count of target organisms on the seed prior to planting.

safer microgreens and possibly other soil grown plants. Harvesting microgreens further away from the soil surface could be beneficial for avoiding possible contamination.

Results from both studies indicated that harvest period and production practices played a major role on the growth and survival of *Salmonella* and STEC on microgreens. For harvest period, counts of both target organisms on all microgreen plants were reduced as time progressed, suggesting it is essential to prolong harvest period. Furthermore, even though reduction was observed for all three microgreens, it appeared that clover showed a greater reduction compared to broccoli and mustard. Additionally, as for production practices, *Salmonella* and STEC on both broccoli and clover microgreens were present at significantly lower levels when cutting at 6.5 cm compared to 2.5 cm, indicating that cutting practices also have an effect on bacterial survival in microgreens.

Evaluation of Plant Type on the Growth and Survival of Salmonella and STEC on Microgreens

On behalf of the production practices study, broccoli, clover, and mustard microgreens were compared statistically to determine if these three types of microgreens share similarities in pathogen growth and survival of *Salmonella* and STEC. If so, it is hypothesized, that it could it be based on their plant type.

Salmonella. Average counts of *Salmonella* on broccoli, clover, and mustard microgreens at 2 weeks of growth for different inoculum levels is shown in Figure 4. At 2 weeks of microgreen growth, for levels starting at 1 and 3 logs, broccoli and clover contained similar bacterial counts, but mustard contained lower counts ($P<0.05$). For concentrations starting at 8 logs, counts on clover microgreens were similar to those on

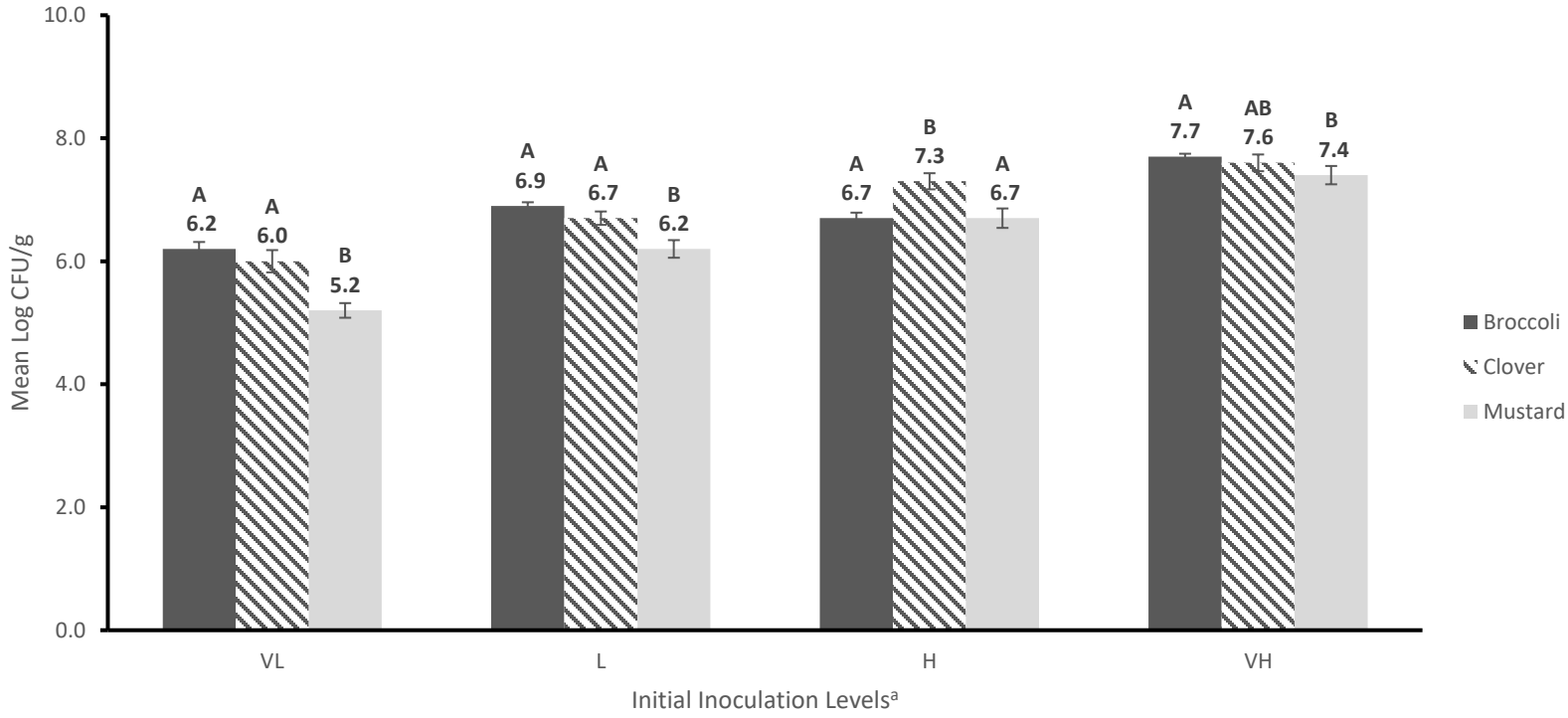

Figure 4. Comparison of *Salmonella* counts (log CFU/g)[b] for all commodity types at two weeks

[a] Initial Inoculation Levels (1(VL), 3(L), 5(H) and 8(VH) log) refers to the target log concentration on the contaminated seeds.

[b] Columns lacking same letter are significantly different ($P<0.05$). Vertical bars represent standard error (n=3).

both broccoli and mustard microgreens. Moreover, at 2 weeks, it appeared all commodities contained similar bacterial counts for different concentration levels; which is why no major differences of bacterial counts were observed between microgreens. This could be because at 2 weeks most of the microgreens had their seed coats still attached to the plant.

Mean counts of *Salmonella* on all commodities inoculated at different concentration levels and cultivated for four weeks are shown in Figure 5. For initial concentrations of 1, 3, and 8 logs, broccoli and mustard had similar levels of *Salmonella* and were significantly different from clover by 1.5 log ($P<0.05$). For inoculation levels of 5 logs, there was no significant difference observed between all microgreens. Furthermore, it would appear at 4 weeks of growth, mustard and broccoli had similar *Salmonella* counts for majority of the concentration levels compared to clover. The similarly high bacterial counts for broccoli and mustard could be based upon a feature observed during their production. For example, at 4 weeks of growth, large numbers of seed coats for broccoli and mustard were still present on their leaves, whereas clover had few present, as previously shown in Figure 3. This could be because both broccoli and mustard typically have the same harvest time (7 to 12 d after germination), whereas clover has a shorter harvest time (6 to 9 d after germination). The early plant development for clover microgreen might explain why clover loses its seed coats faster than mustard and broccoli microgreens. Therefore, this may also explain why both broccoli and mustard shared similar bacterial counts.

STEC. Mean counts of STEC inoculated at different levels on all microgreens and then allowed 2 weeks to grow is shown in Figure 6. For levels with concentrations starting

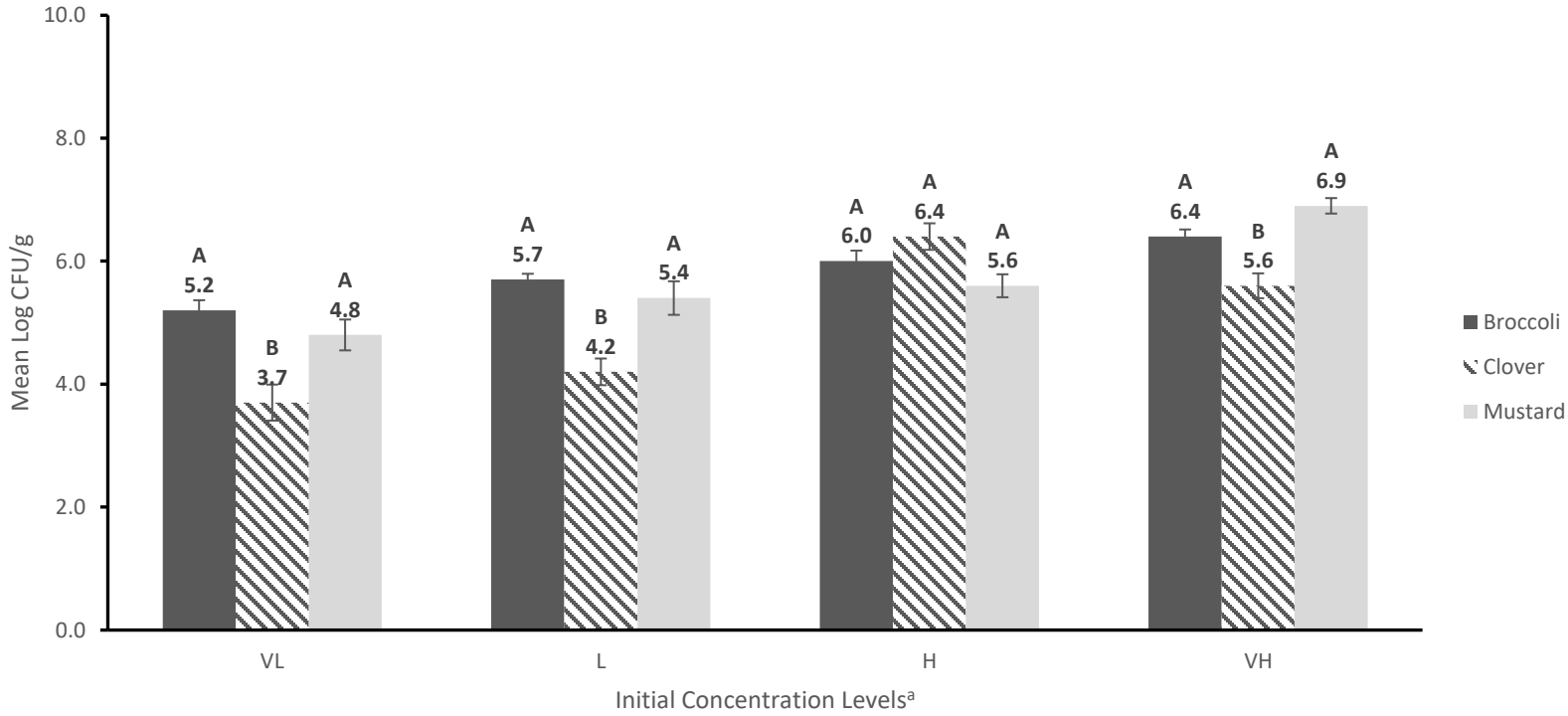

Figure 5. Comparison of *Salmonella* counts (log CFU/g)[b] for all commodity types at four weeks

[a] Initial Concentration Levels (1(VL), 3(L), 5(H) and 8(VH) log) refers to the target log concentration on the contaminated seeds.

[b] Columns lacking same letter are significantly different ($P<0.05$). Vertical bars represent standard error (n=3).

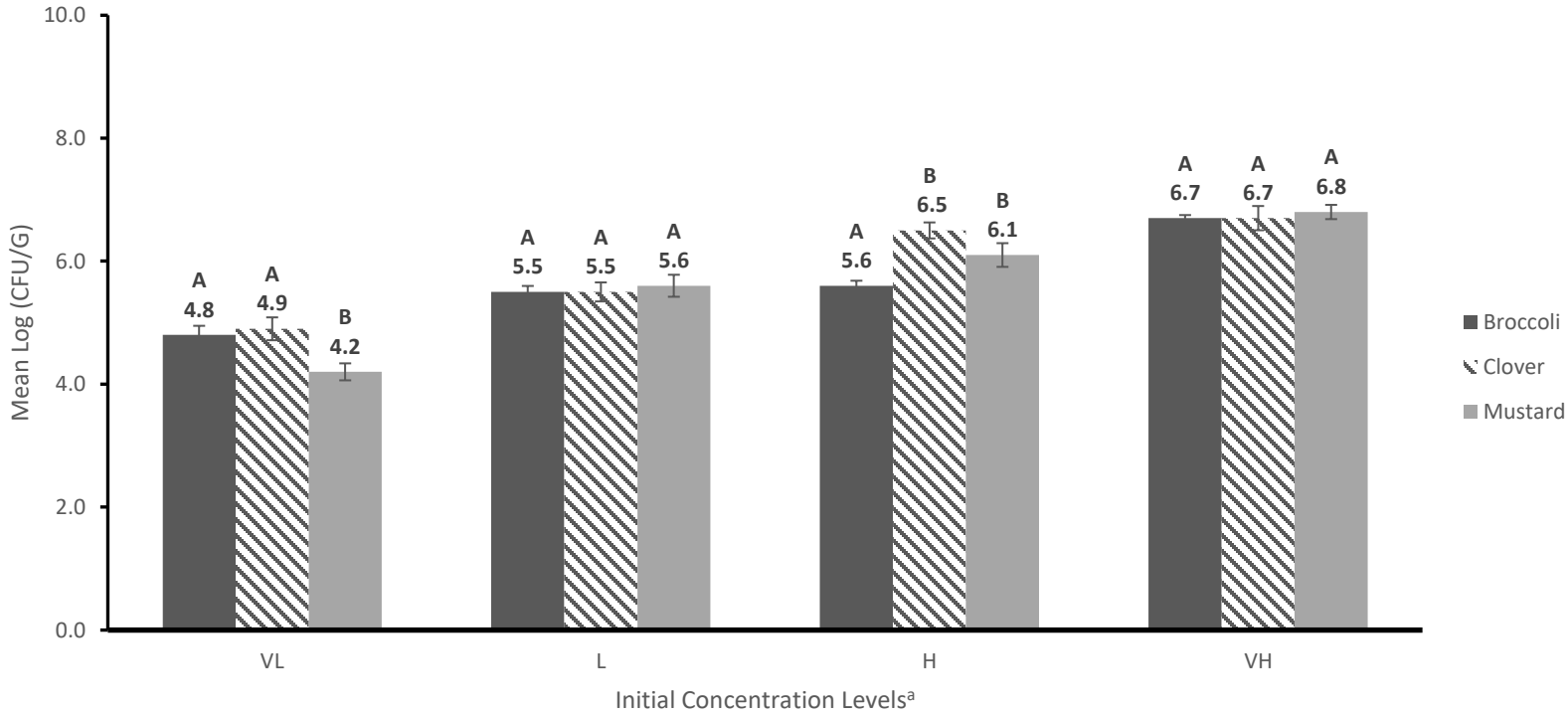

Figure 6. Comparison of STEC counts (log CFU/g)[b] for all commodity types at two weeks

[a] Initial Concentration Levels (1(VL), 3(L), 5(H) and 8(VH) log) refers to the target log concentration on the contaminated seeds.

[b] Columns lacking same letter are significantly different ($P<0.05$). Vertical bars represent standard error (n=3).

at 1 log, broccoli and clover were different from mustard microgreens by as much as 0.7 logs after two weeks. At inoculation levels of 5 logs, clover and mustard were different from broccoli by 0.9 logs ($P<0.05$) at week 2 of observation. There was no significant difference observed between 3 and 8-log inoculation levels for all three microgreens plants. Similar to *Salmonella*, at two weeks of growth, no major difference was observed between STEC counts for all microgreens for all inoculum levels, possibly because most of the seed coats were still attached.

During week 4 of growth, average counts of STEC on all three commodities inoculated at different concentration levels is shown in Figure 7. For initial concentrations of 1, 3, and 8 logs, broccoli and mustard had similar STEC counts and were significantly different from clover by 1.4 log ($P<0.05$). Similar to *Salmonella,* at four weeks of growth, mustard and broccoli had similar STEC counts for most of the concentration levels when compared to clover. As previously stated, through observation during 4 weeks of growth, large numbers of seed coats for broccoli and mustard were still present on their leaves, whereas clover had little to none present, as previously shown in Figure 3.

In general, the results indicated that plant types may play a role in the growth and survival of target pathogens. For instance, when harvesting at 2 weeks, broccoli, clover, and mustard had majority of seed coats attached to their leaves, which would explain why there were no major differences for bacterial counts observed between the three plants at that time. However, at 4 weeks of growth, broccoli and mustard had similar counts for both *Salmonella* and STEC for the majority of inoculation levels. Both broccoli and mustard microgreens contained higher concentrations of both *Salmonella* and STEC

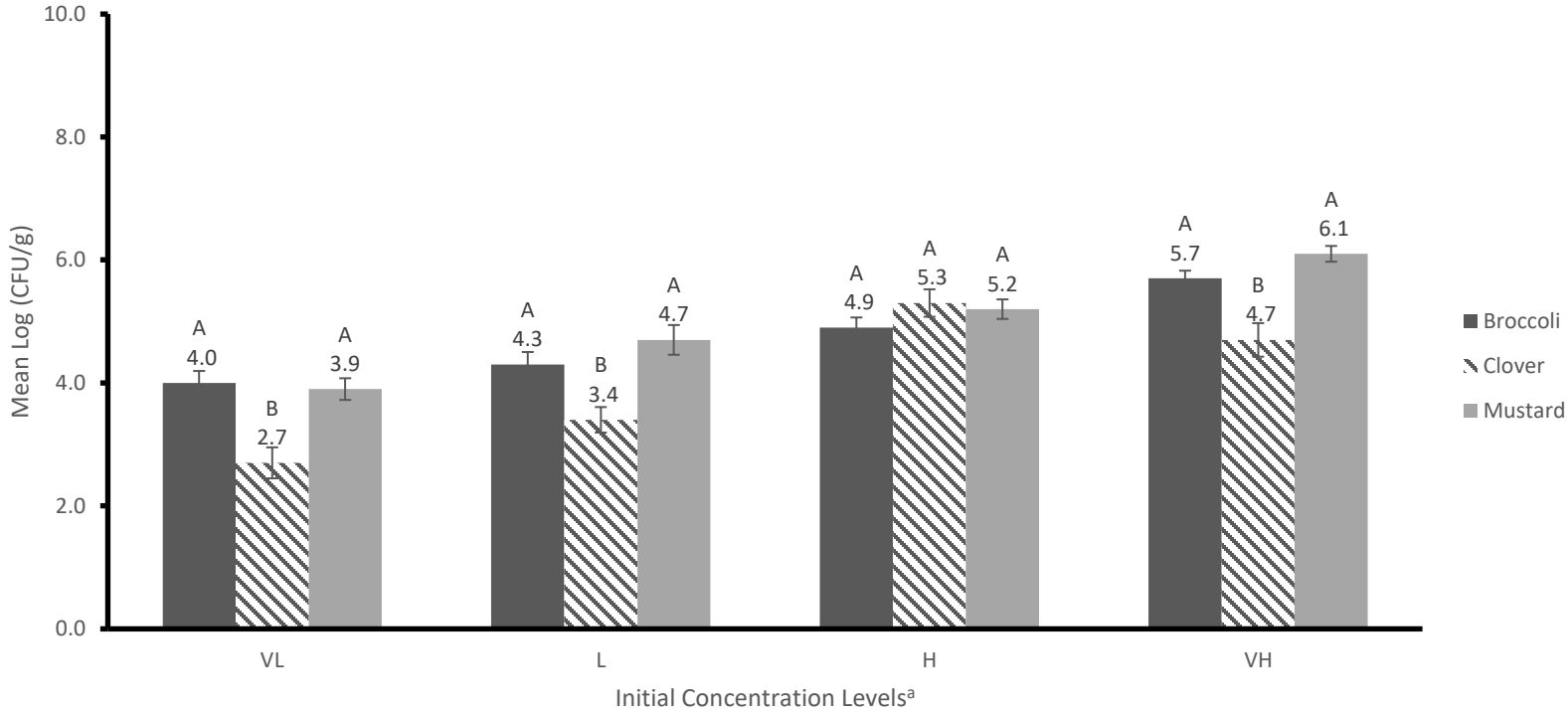

Figure 7. Comparison of STEC counts (log CFU/g)[b] for all commodity types at four weeks

[a] Initial Concentration Levels (1(VL), 3(L), 5(H) and 8(VH) log) refers to the target log concentration on the contaminated seeds.

[b] Columns lacking same letter are significantly different ($P<0.05$). Vertical bars represent standard error (n=3).

organisms compared to clover microgreens, likely because broccoli and mustard had more seed coats still present on their leaves at 4 weeks, while clover microgreens had shed most of the seed coats. Another explanation through observation, as mentioned previously, could have to do with the harvest time of the different seed types. For instance, in this study broccoli and mustard microgreens typically had the same harvest period (7 to 12 d after germination), whereas clover microgreens had a shorter harvest period (6 to 9 d after germination). The early plant development for clover microgreens might be the reason why clover sheds its seed coats earlier than mustard and broccoli microgreens. Furthermore, this may also explain why both broccoli and mustard shared similar bacterial counts than compared to clover microgreens.

In conclusion, the data presented in this objective would indicate seed coats could be the primary source for microgreen contamination. For instance, supporting the theory of the seed coats being a major point of contamination, a small preliminary study was conducted to determine counts of target organisms on seed coats and the leaves. Results indicated seed coats contained up to 7.0 log CFU/g, while leaves contained only 2.3 log CFU/g of both target organisms. This 4.7-log difference between the seed coat and leaves indicates seed coats are likely a primary source of contamination. In order to be able to pin-point the seed coats as the primary source for microgreen contamination, further observational research is required to validate the theory that seed coats are the main point of contamination. The use of the confocal laser-scanning microscopy will be used in the later study to determine the microbial distribution of target organism on microgreens (edible or inedible portions).

Evaluation of *S.* Poona Distribution of Microgreens Using a Confocal Microscope

The purpose of this portion of the study was to examine the distribution of *S.* Poona on the edible and inedible portions of microgreen plants at different harvest periods to determine from previous studies if the seed coat is the main point of contamination. After testing soil in our previous study, results revealed seed coats may be the primary source of contamination for microgreens. Therefore, for this study, it is hypothesized that the inedible sections of the microgreens in particular, seed coats could be the main point of contamination.

Edible Portions

S. Poona RFP, which fluorescent with bright red circular colonies against the dark background of microgreen tissue, was present in all sections of mustard and clover microgreens from heavily populated to minimally populated. The edible portions as previously mentioned consisted of leaves and middle shoot (7.5 and 6.5 cm). The leaves for mustard and clover had minimal presence of *S.* Poona at 2 and 4 weeks are shown in Figure 8-11. Visual counts of bacteria on leaves and the middle shoot of clover and mustard microgreens at 2 and 4 weeks is presented in Table 11. The average counts of *Salmonella* on mustard leaves at 2 and 4 weeks were 0.2 log CFU/μm^2. For clover leaves, average counts of *Salmonella* at 2 and 4 weeks of growth were 0.5 and 0.2 log CFU/μm^2, respectively. This indicated a 0.3-log difference observed between 2 and 4 weeks for clover leaves, and no differences were observed for mustard leaves at 2 and 4 weeks. This would suggest that contamination of the leaves is very minimal, and if contamination occurred it is possibly due to cross-contamination.

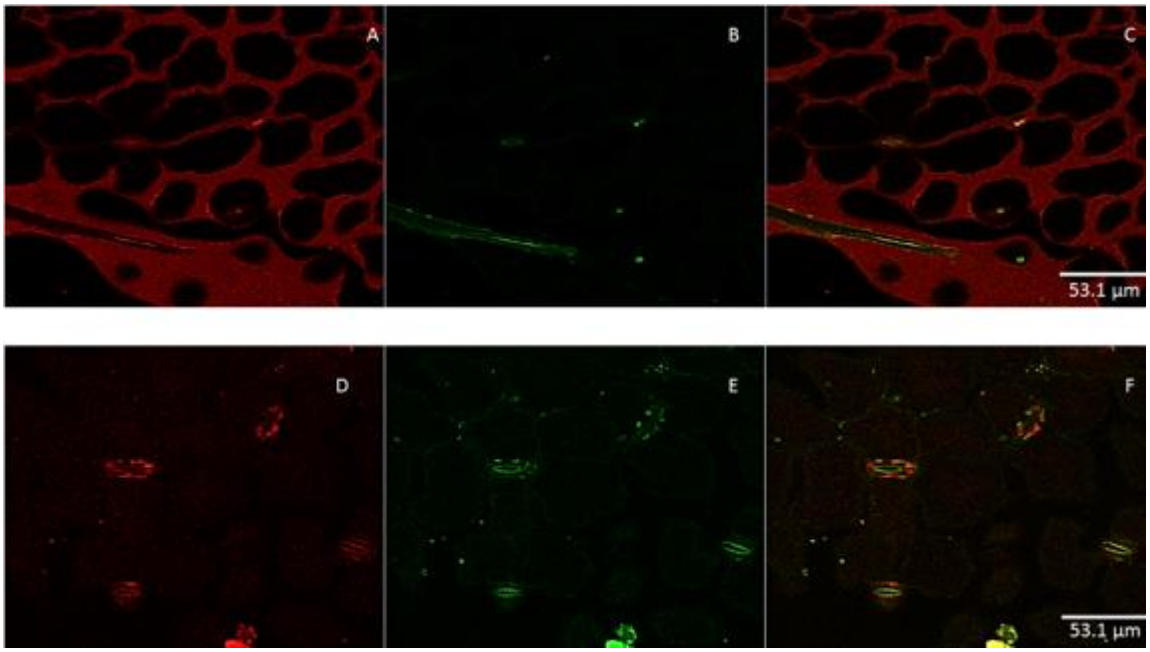

Figure 8. *S.* Poona distribution on clover (leaf) at two weeks using confocal microscope (A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated leaf (7.5 cm above soil surface).
(D) Red, (E) Green, and (F) Red and Green together represents the inoculated leaf (7.5 cm above soil surface).
All images were taken using a three-dimensional screening.

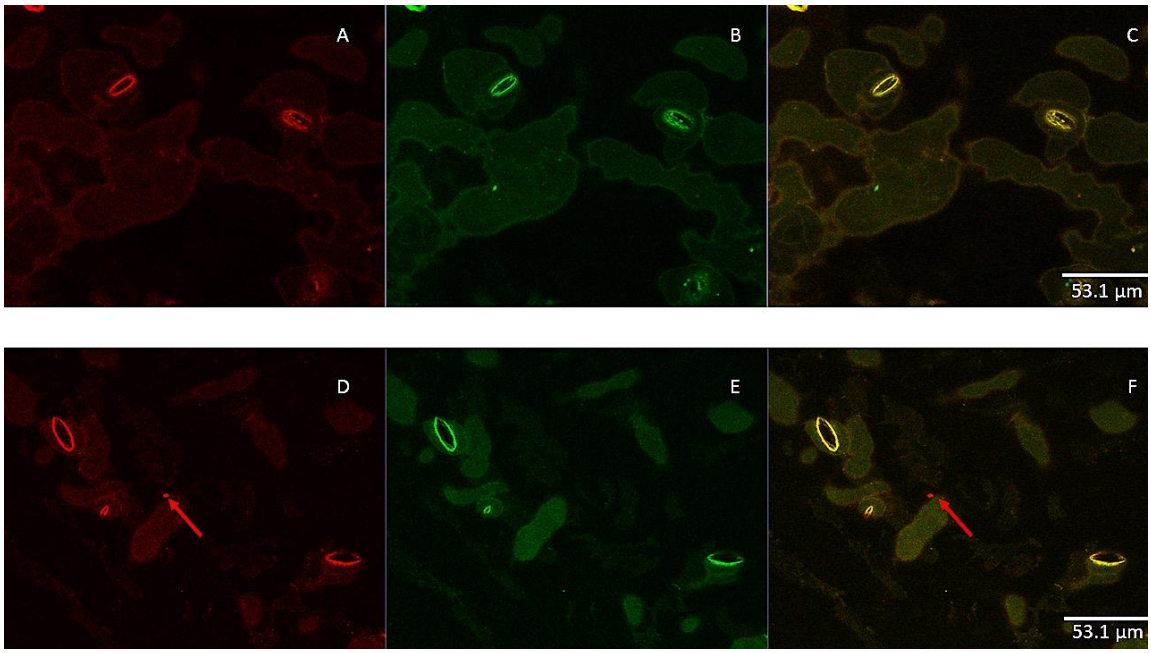

Figure 9. *S.* Poona distribution on mustard (leaf) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated leaf (7.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated leaf (7.5 cm above soil surface).

All images were taken using a three-dimensional screening.

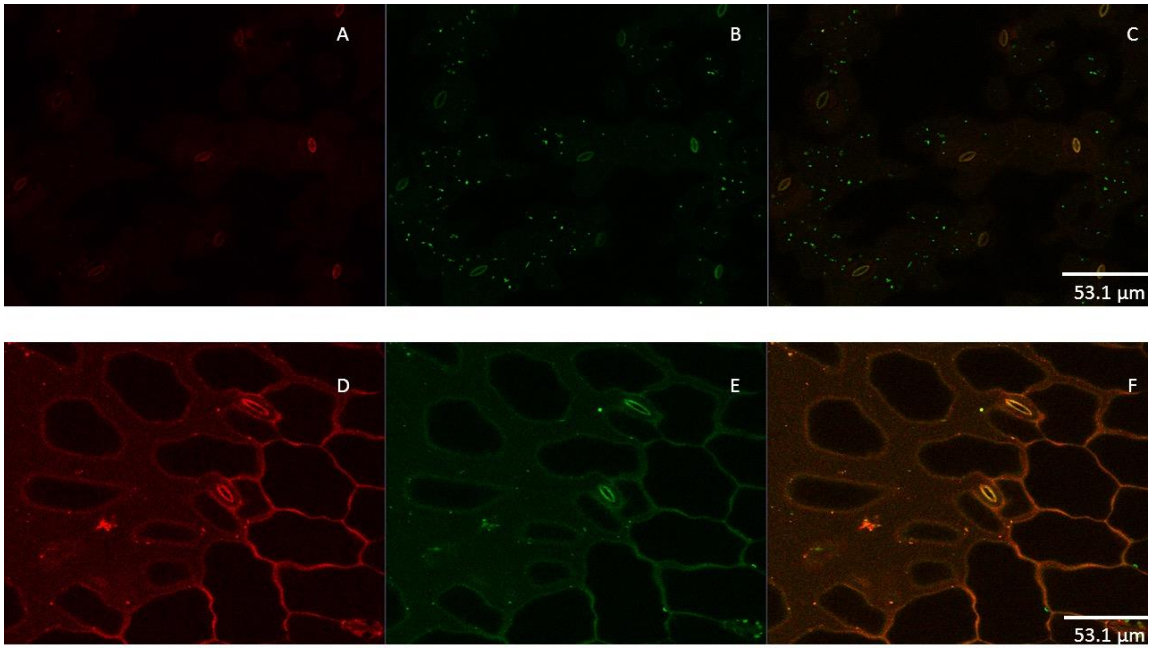

Figure 10. *S.* Poona distribution on clover (leaf) at four weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated leaf (7.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated leaf (7.5 cm above soil surface).

All images were taken using a three-dimensional screening.

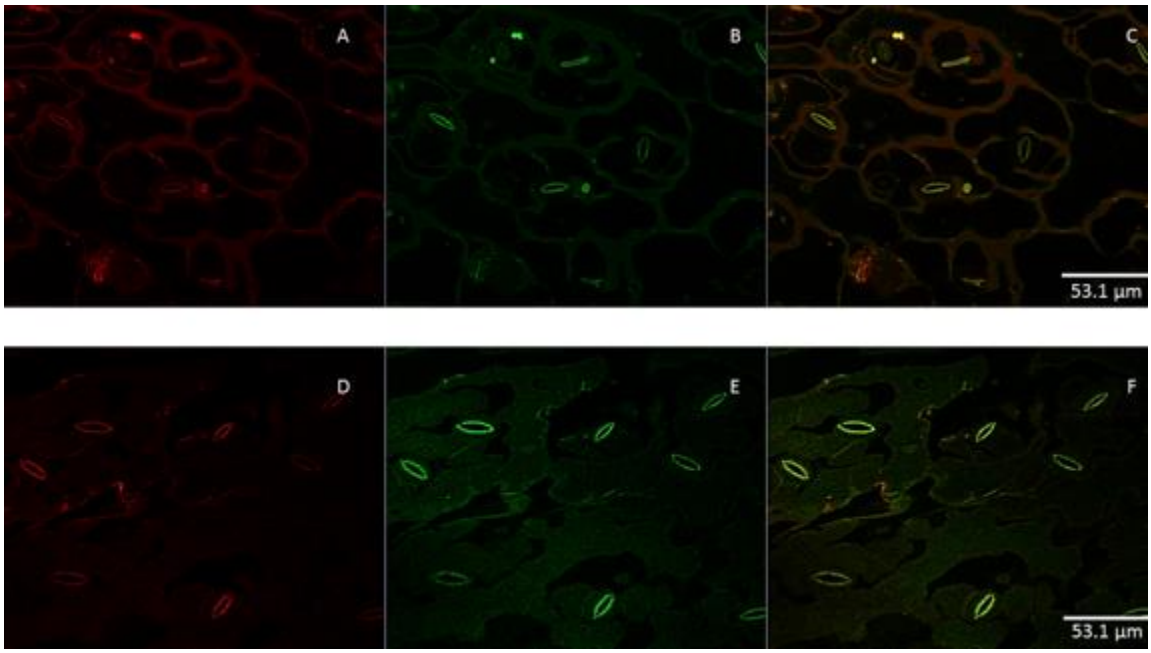

Figure 11. *S.* Poona distribution on mustard (leaf) at four weeks using confocal microscope
(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated leaf (7.5 cm above soil surface).
(D) Red, (E) Green, and (F) Red and Green together represents the inoculated leaf (7.5 cm above soil surface).
All images were taken using a three-dimensional screening from 1µm section.

Table 11. Mean counts (log CFU/μm^2)[a] of *S.* Poona on edible portions of microgreens

Microgreens					
	Edible Portion				
Microgreen	**Section**	**SEM**	**2 Weeks**	**SEM**	**4 Weeks**
Mustard	Middle Shoot (6.5 cm)	0.500	1.0^{A}	0.352	0.7^{A}
Mustard	Leaf (7.5 cm)	0.086	0.2^{A}	0.150	0.2^{A}
Clover	Middle Shoot (6.5 cm)	0.100	0.4^{A}	0.213	0.6^{A}
Clover	Leaf (7.5 cm)	0.213	0.5^{A}	0.150	0.2^{A}

[a] Mean within a row, at each time, and with different superscripts are significantly different ($P<0.05$)

At 2 weeks of growth, more cells appeared on the middle shoot (6.5 cm) for mustard and clover microgreens, compared to leaves (Figure 12 and 13); however, at 4 weeks few cells were detected as shown in Figure 14 and 15. Average visual counts of *Salmonella* on middle portions of mustard and clover microgreens are presented in Table 10. Mean counts of *Salmonella* on middle sections of mustard microgreens were 1.0 and 0.7 log CFU/μm^2 at 2 and 4 weeks, respectively. Mean counts of *Salmonella* on the middle shoot of clover at 2 and 4 weeks were 0.4 and 0.5 log CFU/μm^2, respectively. There was no significant difference between contamination of leaves and middle portions of both clover and microgreens at 2 and 4 weeks ($P>0.05$); however, a significant difference was observed between edible and inedible portions of both commodities at 2 and 4 weeks ($P<0.05$). Inedible portions contained higher counts of *Salmonell*a than edible portions by as much as 2.2 log CFU/g for clover microgreens and 1.2 log CFU/g for mustard microgreens. This would suggest that leaves and middle section are the least populated sections of microgreens compared to inedible portions. If contamination occurs on leaves, it is likely caused by contact with the exterior surface of the seed coats through cross-contamination, since interior surfaces of the seeds are thought to be sterile *(132).* Furthermore, if contamination occurs on middle portions of the stem it is likely that seeds are spread densely prior to planting, and when the seed coats falls from leaves it may come into contact with the middle shoot causing contamination.

Inedible Portions

The inedible portions of the microgreens included the lower shoot (2.5 cm) and seed coats. In comparison to the edible portions, the inedible portions at 2 weeks were

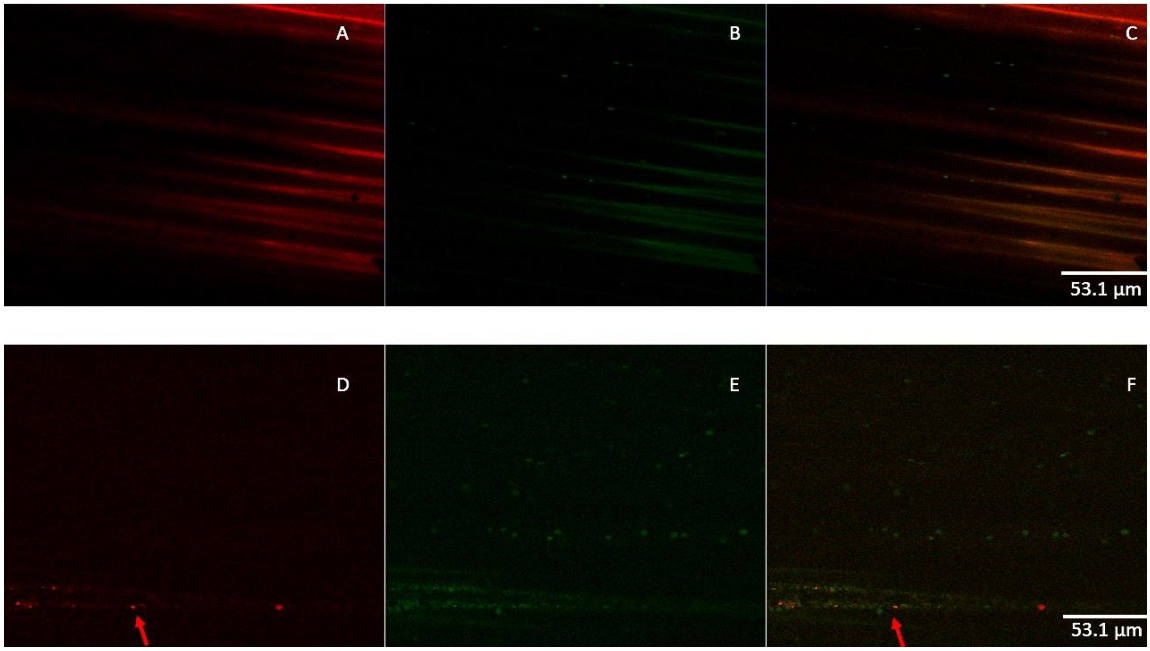

Figure 12. *S.* Poona distribution of clover (middle) at two weeks using confocal microscope (A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated middle shoot (6.5 cm above soil surface).
(D) Red, (E) Green, and (F) Red and Green together represents the inoculated middle shoot (6.5 cm above soil surface).
All images were taken using a three-dimensional screening. Arrows represents target organism.

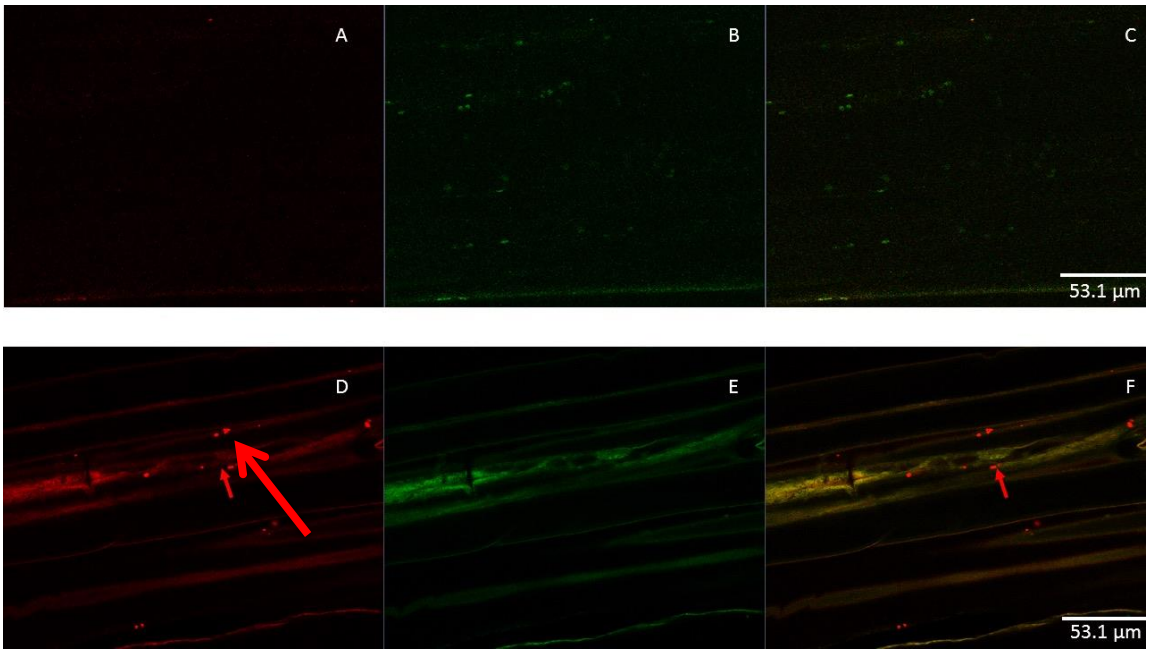

Figure 13. *S.* Poona distribution of clover (middle) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated middle shoot (6.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated middle shoot (6.5 cm above soil surface).

All images were taken using a three-dimensional screening. Arrows represents target organism.

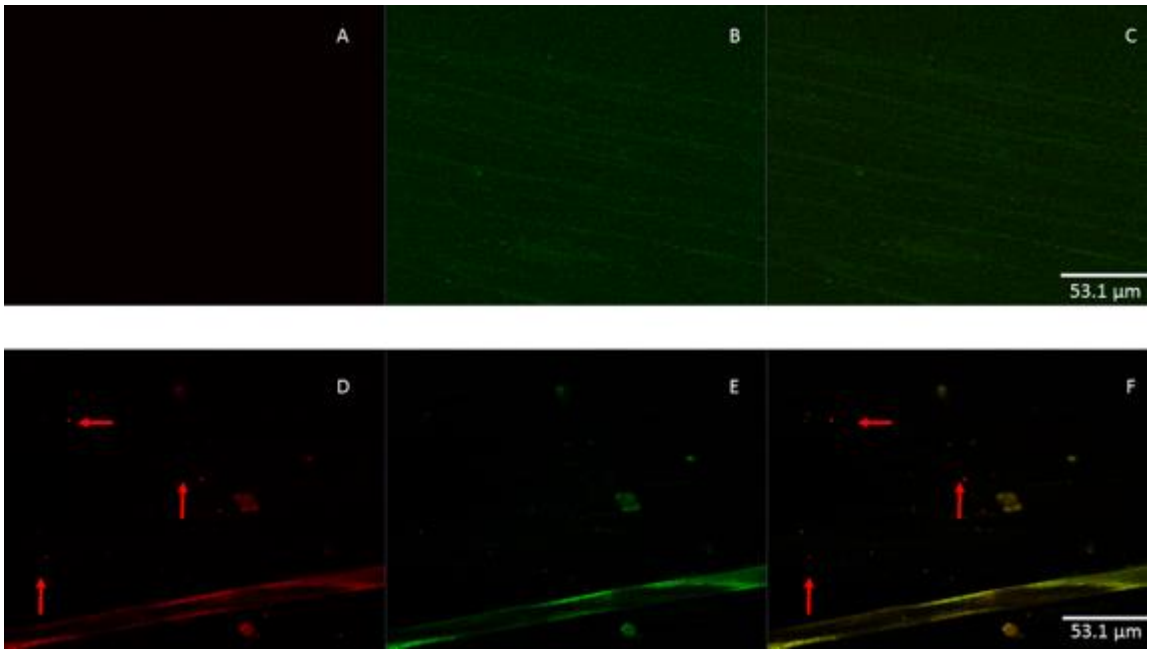

Figure 14. *S.* Poona distribution of clover (middle) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated middle shoot (6.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated middle shoot (6.5 cm above soil surface).

All images were taken using a three-dimensional screening from 1µm section. Arrows represents target organism.

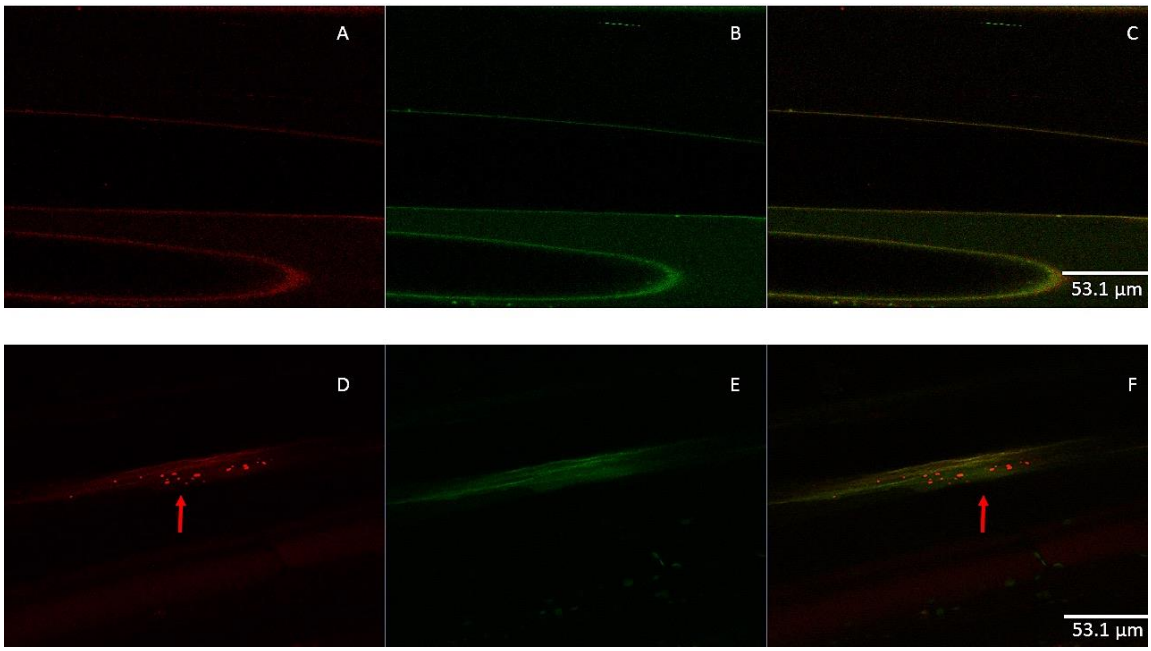

Figure 15. *S.* Poona distribution of clover (middle) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated middle shoot (6.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated middle shoot (6.5 cm above soil surface).

All images were taken using a three-dimensional screening from 1µm section. Arrows represents target organism.

heavily populated with *S.* Poona for both clover and mustard microgreens. Visual counts of *Salmonella* on mustard and clover inedible portions are presented in Table 12. The lower shoot (2.5 cm) was more populated with target organism compared to the middle stem and leaves for both mustard and clover at 2 weeks as shown in Figure 16-19. Average counts of *Salmonella* on mustard and clover lower shoots at 2 weeks were 1.6 and 2.4 log CFU/μm^2. In addition, at 4 weeks mean counts for mustard and clover were 0.8 and 0.9 log CFU/μm^2.

The seed coats as shown in Figure 20-23 were also heavily populated with *S.* Poona at 2 and 4 weeks for both mustard and clover microgreens. The number of cells per image were too numerous to count (TNTC) for both commodities at both time points, indicating that seed coats remained the main point of contamination throughout the harvest period for both mustard and clover microgreens. A similar trend was also observed when Xiao et al. *(235)* viewed *E. coli* O157:H7 GFP on radish microgreens. They observed both the lower shoot and seed coats were heavily populated with *E. coli* O157:H7 GFP; however, the seed coats remained the main point of contamination in their study. Although their study reported similar results to this research, they only collected data for 1 week while the present study used two time points, providing a unique contribution to the limited data published on the subject.

This portion of the study analyzed *S.* Poona RFP distribution on mustard and clover microgreens throughout the harvest period. Results indicated that contamination remained higher on seed coats throughout the harvest period for both commodities. As mentioned previously, similar findings were observed when Xiao et al. *(235)* viewed *E. coli* O157:H7

Table 12. Mean counts (log CFU/μm^2) of *S.* Poona on inedible portions of microgreens

Microgreens					
		Inedible Portion			
Microgreen	**Section**	**SEM**	**2 Weeks**	**SEM**	**4 Weeks**
Mustard	Lower Shoot (2.5 cm)	0.000	1.6	0.283	0.8
Mustard	Seed Coat	-	TNTC	-	TNTC
Clover	Lower Shoot (2.5 cm)	0.284	2.4	0.392	0.9
Clover	Seed Coat	-	TNTC	-	TNTC

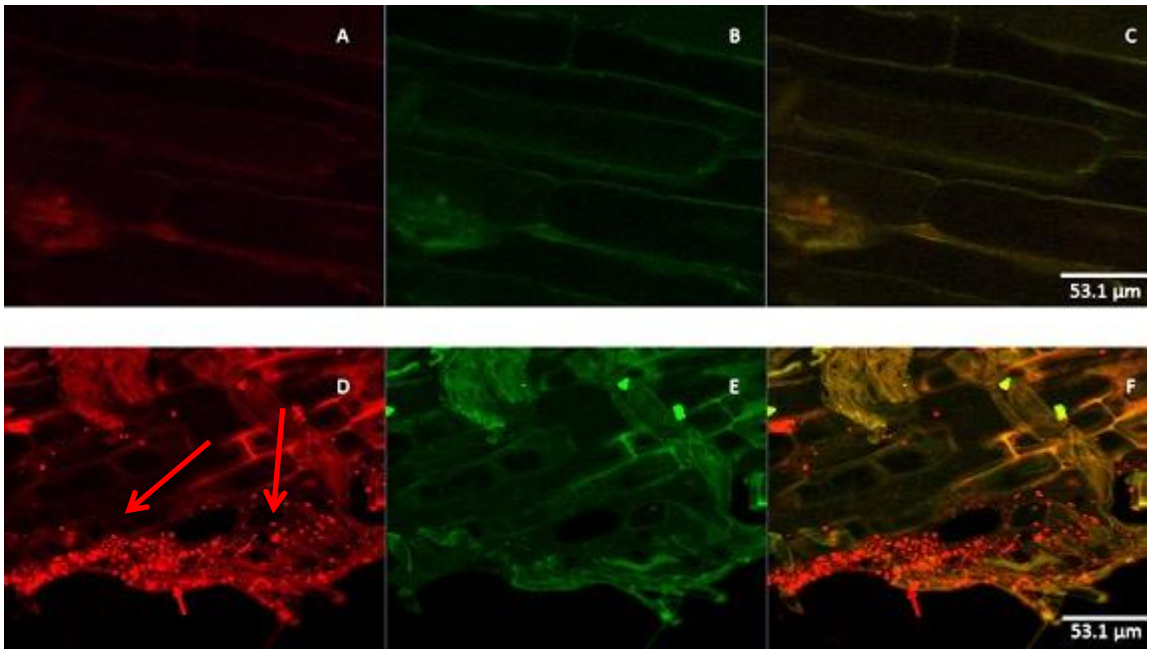

Figure 16. *S.* Poona distribution on clover (lower) at two weeks using confocal microscope (A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated lower shoot (2.5 cm above soil surface).
(D) Red, (E) Green, and (F) Red and Green together represents the inoculated lower shoot (2.5 cm above soil surface).
All images were taken using a three-dimensional screening. Arrows represents target organism.

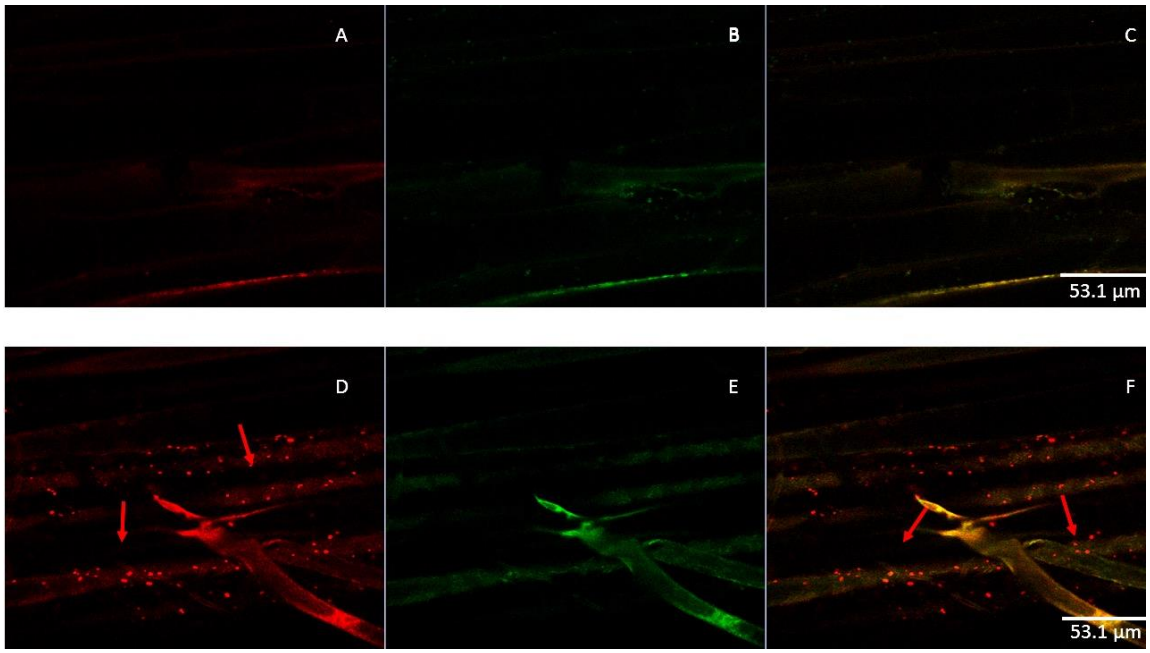

Figure 17. *S.* Poona distribution on mustard (lower) at two weeks using confocal microscope
(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated lower shoot (2.5 cm above soil surface).
(D) Red, (E) Green, and (F) Red and Green together represents the inoculated lower shoot (2.5 cm above soil surface).
All images were taken using a three-dimensional screening. Arrows represents target organism.

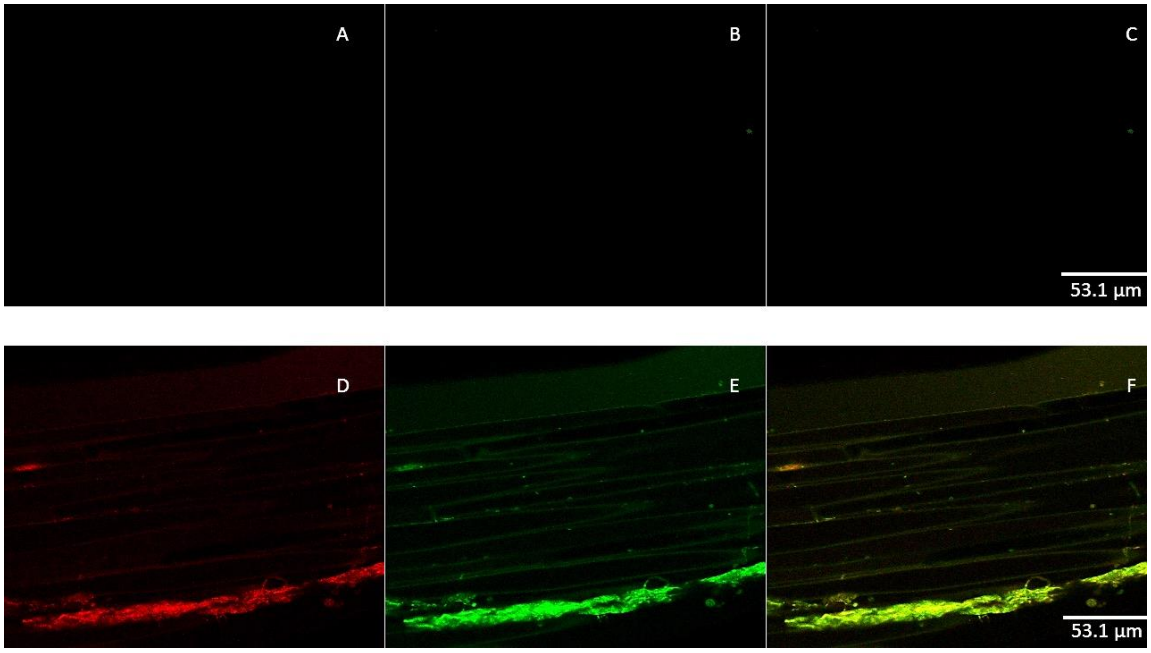

Figure 18. *S.* Poona distribution on clover (lower) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated lower shoot (2.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated lower shoot (2.5 cm above soil surface).

All images were taken using a three-dimensional screening.

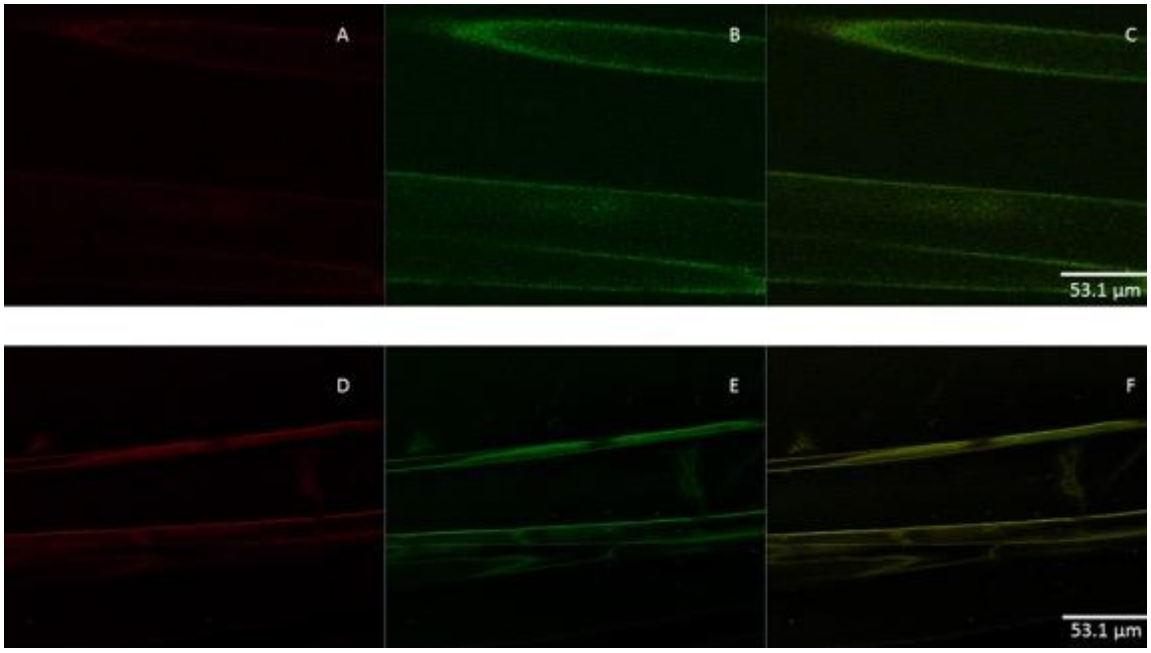

Figure 19. *S.* Poona distribution on mustard (lower) at four weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated lower shoot (2.5 cm above soil surface).

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated lower shoot (2.5 cm above soil surface).

All images were taken using a three-dimensional screening.

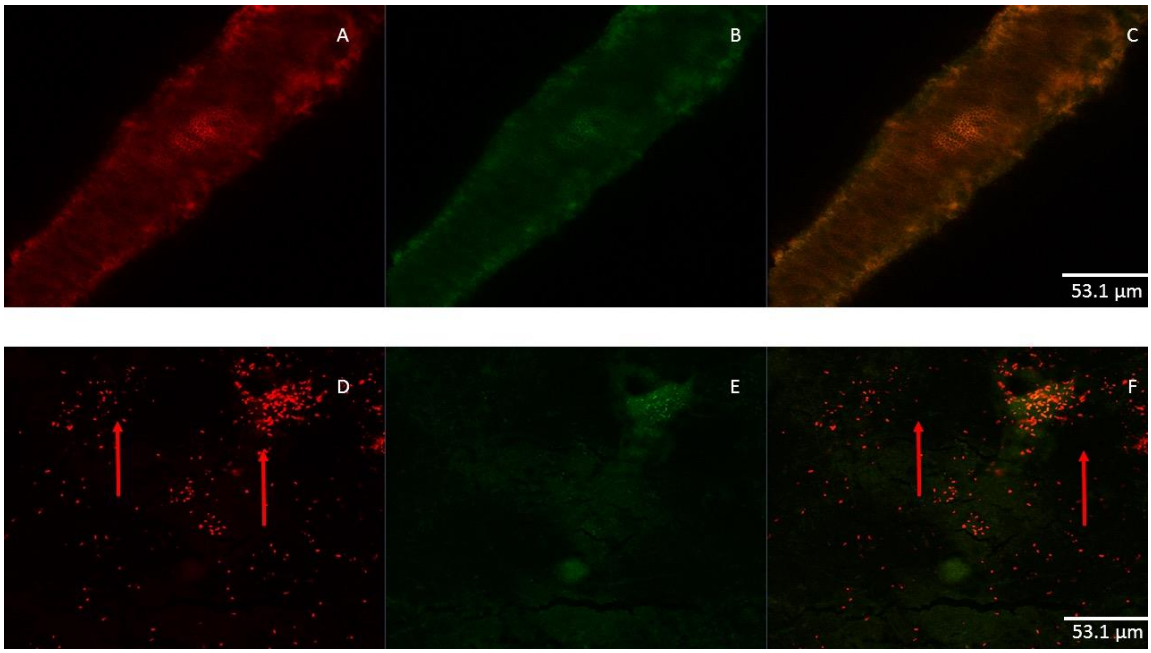

Figure 20. *S.* Poona distribution on clover (seed coat) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated seed coat.

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated seed coat.

All images were taken using a three-dimensional screening. Arrows represents target organism.

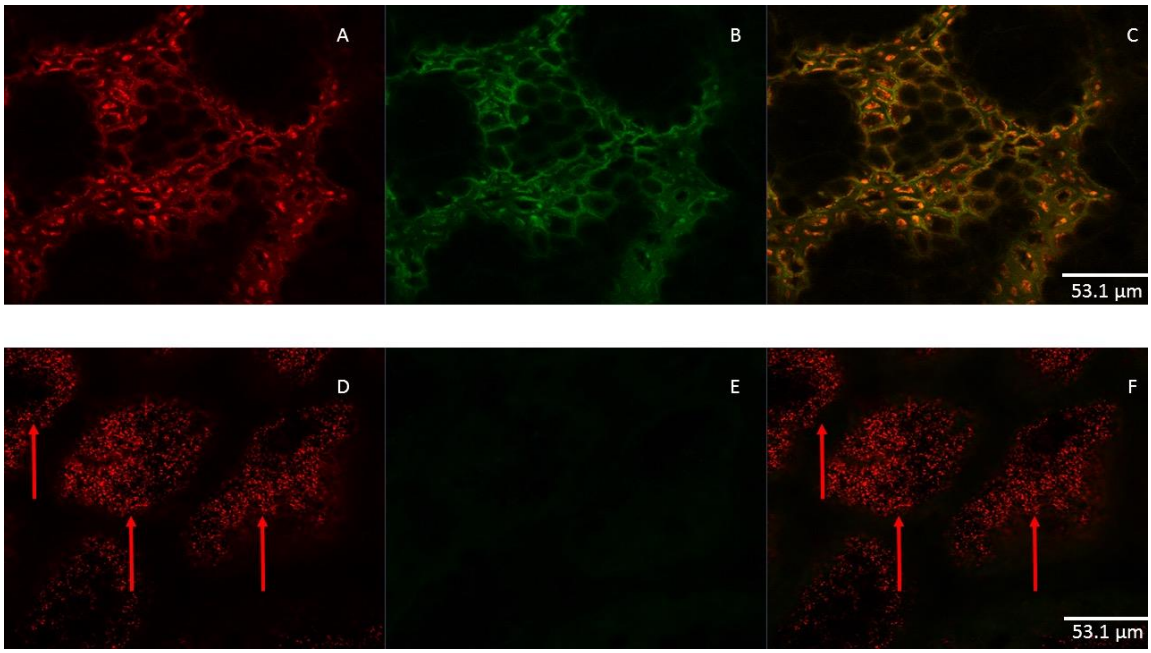

Figure 21. *S.* Poona distribution on mustard (seed coat) at two weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated seed coat.

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated seed coat.

All images were taken using a three-dimensional screening. Arrows represents target organism.

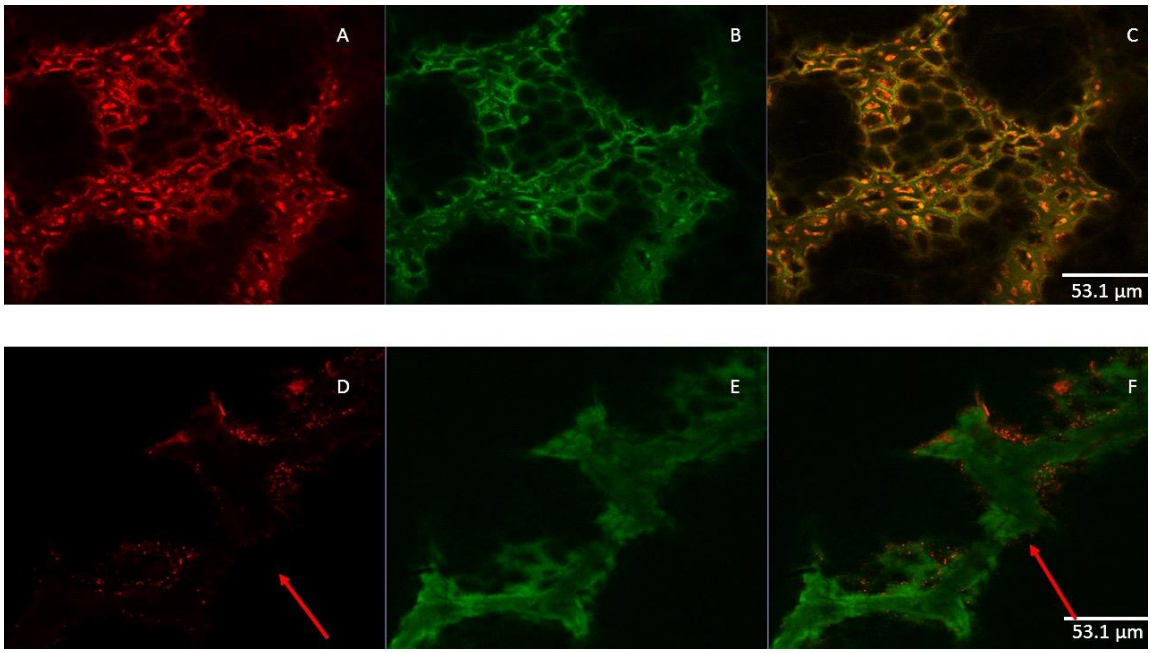

Figure 22. *S.* Poona distribution on mustard (seed coat) at four weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated seed coat.

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated seed coat.

All images were taken using a three-dimensional screening. Arrows represents target organism.

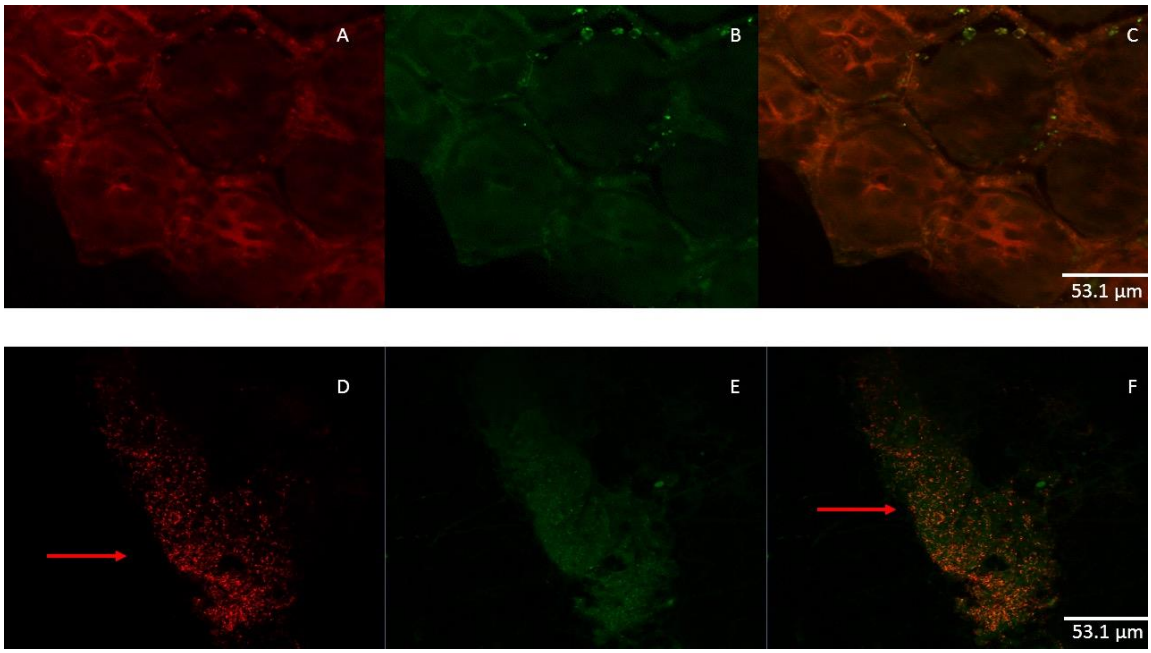

Figure 23. *S.* Poona distribution on clover (seed coat) at four weeks using confocal microscope

(A) Red, (B) Green, and (C) Red and Green together represents the non-inoculated seed coat.

(D) Red, (E) Green, and (F) Red and Green together represents the inoculated seed coat.

All images were taken using a three-dimensional screening. Arrows represents target organism.

on different portions of radish microgreens, and reported that seed coats were the main source of contamination. For this study, although contamination could be observed mainly on the inedible portions of both mustard and clover microgreens, middle sections and leaves also had little contamination. This contamination could occur when contaminated seed coats fall from the leaves and contacts the lower stem, causing cross-contamination. Also, if seeds are spread densely prior to planting, the middle shoot may have contact with the contaminated seeds.

Leaves of both mustard and clover microgreens were observed to be the least populated section of the microgreens. This could be because when seed coats are attached to the tip of the leaf, they are attached through the interior opening of the seed coat that is thought to be sterile *(132)*, and when released, minimal contact between contaminated seed and leaf occurs. Therefore, if contamination occurs, it is likely caused by contact with the exterior surface of the seed coats through cross-contamination.

This study can be used as a validation of what was observed in the previous studies (Objective 2). As seen in production practices, by harvesting higher up the stem, the chances of contamination became lower. Also discussed earlier, as growth time increased, fewer cells were detected. Finally, the theory that seed coats were a main point of contamination in previous studies was validated through this observational study. Regardless of time or commodity, seed coats remained more populated with target organisms when compared to any other portion of the plant. Therefore, this study can conclude that the seed coats are a focal point of contamination for both microgreens.

CHAPTER V

CONCLUSIONS

This study investigated the growth of *Salmonella* and STEC on alfalfa sprouts and microgreens, and also compared the two-target organisms for each commodity. Results from this study indicated a significant difference between sprouts and microgreens grown from same contaminated seed. This difference can be due to growth conditions used for sprouts such as high humidity, warm temperatures, and continued water availability. Another possibility for the fewer counts of pathogenic bacteria found on microgreens could be because only part of the plant was harvested whereas the entire plant was used for sprouts. The study also signified that *Salmonella*, regardless of the commodity, were significantly higher STEC concentrations. This finding would suggest that *Salmonella* could be able to attach better to both sprouts and microgreens compared to STEC. In addition, *Salmonella* could be able to utilize nutrients faster than STEC. The significance of the study indicated sprouts were able to harbor higher concentrations of the target pathogens during sprouting compared to microgreens during germination. Therefore, it is of importance to ensure the safety of the product by minimizing contamination during sprouting and germination processes.

This study also investigated the effect of production practices and plant variety on the growth and survival of *Salmonella* and STEC on microgreens. Harvest period and production practices played a significant role on bacterial growth and survival. At 2 weeks, average counts for *Salmonella* and STEC on microgreens grown from contaminated seeds

increased for all microgreen types, and for all levels of initial inoculum except for VH. Mean counts of *Salmonella* and STEC were significantly lower at 4 weeks for all three microgreens and inoculum levels compared to 2 weeks. In addition, clover contained significantly lower counts compared to broccoli and mustard ($P<0.05$), which could be due to the considerable high amount of seed coats still present on broccoli and mustard during this time. Furthermore, at 6 weeks of growth, counts of both *Salmonella* and STEC on clover were significantly lower than 2 and 4 weeks, even though at this point it is considered as a baby green ($P<0.05$). This continues to support that harvest period (time) plays an important role in the reduction of target organisms. Soil was analyzed to determine the likely source of contamination. Results indicated there was no significant difference of *Salmonella* counts observed between broccoli microgreens and soil for majority of inoculum levels at 2 and 4 weeks. *Salmonella* counts for clover; however, contained significant differences between soil and microgreens for majority of inoculum levels ($P<0.05$). The minimal seed coats present on clover plants during week 4 could be the reason for observing difference in counts between the plant and soil samples. For instance, when the contaminated seed detach from the leaf it will most likely contaminate the soil, and thus leaving little contamination on the actual plant itself. This is also true for broccoli since their seed coats are apparent during harvesting, the counts in the soil would be similar to the counts on the plants. STEC also showed similar trends as *Salmonella* with soil, with clover and to a lower extent for broccoli. The second research component of this study evaluated the effect of production practices on the growth and survival of *Salmonella* and STEC on microgreens. Significant differences were observed between the

two harvest lengths for both broccoli and clover microgreens at 2 and 4 weeks ($P<0.05$). The higher in length of the plant or further away from the soil surface, the less contamination of target organisms were observed. This study signified that production practices also plays role in the survival of target organisms found on plants. Lastly, evaluation of plant variety on the growth and survival of *Salmonella* and STEC was observed. Results revealed that broccoli, clover, and mustard microgreens played a role in the growth and survival of target pathogens. For instance, when harvesting at 2 weeks broccoli, clover, and mustard had majority of seed coats attached to the leaf, which would explain why there was no major difference in bacterial numbers observed at this time between the three commodities. However, at 4 weeks, broccoli and mustard carried similar high concentrations of both target organisms for majority of inoculum levels than compared to clover. This fact revealed that at 4 weeks broccoli and mustard had more seed coats still present on their leaves, which explained why they both had similar counts of target organisms than compared to clover. Overall, the findings from this study revealed that harvest period, production practices, and plant type all played a role in the growth and survival of *Salmonella* and STEC.

The last objective of this study investigated the distribution of *S.* Poona on the edible and inedible portions of microgreen plants at different harvest periods using confocal microscopy. The purpose was to determine if the seed coat was the main point of contamination. The results indicated that edible portions (leaves and middle shoot) for both mustard and clover microgreens at 2 and 4 weeks contained little to no *Salmonella*. This could be because once the seed coats fall off from the leaf, the target organism would

have little to no contact with the plant surface unless cross-contaminated with another plant's seed coat. In addition, the inedible portions (lower shoot and seed coat) had more populated areas of target organism than the leaves. This could be because it is near the soil surface where remaining seed coats reside. However, seed coats throughout the growth period remained heavily populated by *Salmonella* compared to all other sections. This indicated that the seed coats are a main point of contamination for microgreens.

This research demonstrated microgreens contained lower bacterial numbers of *Salmonella* and STEC compared to sprouts due to the different types of production. For instance, sprouts and the conditions in which they are grown in (high humidity, high temperature, and constant water availability) can create a greater food safety risk than compared to microgreens production; however, even though to a lower extent, pathogens were also able to proliferate during microgreen growth. Therefore, the importance of minimizing contamination during these processes is essential, especially since neither process involves a kill step for the elimination of bacterial pathogens. The findings from this research also indicated harvest period, production practices, and plant variety played a significant role on the growth and survival of *Salmonella* and STEC found on microgreens. The extended growth period and the further away from the soil surface for harvesting, showed lower counts of *Salmonella* and STEC on all microgreens. Lastly, not only could this research provide factors that could aid in the safety of microgreens, it was also able to pin-point the main source of contamination for microgreens through the use of a fluorescent microscopy. Therefore, the mechanisms behind the microbial distribution

and attachment seen on microgreens should be further examined to get a better understanding of the interactions occurring between microorganisms and plants.

Application of Findings

The findings from this research provided information that could aid in improving the safety of microgreens for production growers. In addition, these studies provide possible new recommendations for improving production practices, such as extending harvesting time, cutting further away from the soil surface, and plant characteristic in regards to food safety. Important recommendations for microgreens growers besides treating seeds and soil would be to extend the harvest period to the true leaf phase and cutting 6.5 cm above soil surface to avoid possible contamination. Not only does this research provide valuable scientific data on the safety of microgreens, but also other soil grown vegetables that are produced from seeds. Finally, this research has provided a greater understanding of the factors that are involved in bacterial reduction seen not only in microgreens, but other vegetable plants during pre-harvest practices.

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